#12 NW

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Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: David R. Kaplan

Title: METHODS FOR DETECTING AN ANALYTE OF INTEREST USING CATALYZED REPORTER DEPOSITION OF TYRAMIDE

Appl. No.: 09/738,049

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Examiner: Gailene Gabel

Art Unit: 1641

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date below.

Line Gauthier
(Printed Name)

Line Gauthier
(Signature)

October 16, 2002
(Date of Deposit)

DECLARATION OF DR. DAVID R. KAPLAN

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, David R. Kaplan, declare that:

1. I earned a Ph.D. in 1979 from the Center for Immunology, University of Chicago, Chicago, IL, and an M.D. in 1980, also from the University of Chicago, Chicago, IL. I have been engaged in research related to pathology and diagnostic methods for 26 years. A copy of my curriculum vitae is attached hereto. I am currently employed as a Professor in the Department of Pathology, Case Western Reserve University, and as Chief Scientist at Flow-Amp Systems, Ltd., 11000 Cedar Avenue, Cleveland, OH 44106, which is a commercial partner of the assignee of the present application.

2. I have reviewed the instant patent application, and I am familiar with the methods for amplification staining in flow cytometry that are described therein. I have also reviewed and am

familiar with the Office Action mailed July 16, 2002. I have also reviewed Karkmann *et al.*, *J. immunol. Meth.* 230: 113-120, 1999, and Lollini, electronically published in volume 1 of *Immunological Blackboard.*

3. The skilled artisan would interpret the Karkmann *et al.* publication as not providing any demonstration that the staining observed (Fig. 1, page 117) is specific to an antigen of interest. Cells were stained with fluoresceinated antibodies to 2 different cytokines (interferon-γ and interleukin 4). Staining was inhibited using the same antibodies lacking a fluorescent label. This control does not validate the specificity of the staining. Instead it indicates the identity of the binding specificity of the unconjugated and conjugated antibody preparations. Thus, while the Karkmann *et al.* publication indicates that certain cells (6-7% of the cells in one case and 2-3% of the cells in another case) bind the labeled antibody, the specificity of this binding has not been demonstrated.

4. Moreover, since antibodies adhere to some types of cells in a manner that is not dependent on the antigenic specificity of the antibody, it is particularly important to verify the specificity of binding especially in circumstances that give an exceedingly small proportion of the cells staining positive with heterogeneous intensity. Verification of specificity can be ascertained by inhibition with soluble cytokines, by the use of nonspecific isotype/subtype matched immunoglobulin controls, by the inhibition of cytokine production during culture with an appropriate cytokine production inhibitor such as cyclosporin A, and/or by the staining of unstimulated cells. Preferrably, more than one of these controls would be used.

5. The Karkmann *et al.* publication also uses a method for assessing fold enhancement that is flawed. By gating a minority of the cells as positive and comparing two different staining and processing protocols, it is not possible to tell which events are corresponding in the comparison groups. In other words, it is impossible to determine which events in the staining in panel A, Figure 1, page 117 correspond with which events in the staining in panel B. The skilled artisan would consider it likely that the amplification procedure caused an inappropriately high level

staining in a subset of cells that accounts for the increase in mean fluorescent values. Without comparisons between homogeneous populations of cells, it is impossible to determine a correct level of fold enhancement provided by these data.

6. Furthermore, I have also determined in my laboratory that the conditions disclosed in the Karkmann *et al.* publication for tyramide staining of intracellular analytes (phosphate buffered saline with 0.5% bovine serum albumin) do not provide specific staining or a 10-fold enhancement of signal in comparison to standard flow cytometry methods when isotype/subtype matched nonspecific immunoglobulin is used as a negative control, due to nonspecific background obtained when using the disclosed conditions.

7. Similarly, while the Lollini publication does compare a homogeneous population of cells, the skilled artisan would understand that the failure to use proper controls in making the measurements renders any claim of specific staining or a 10-fold enhancement of signal unfounded. The Lollini publication compares cells contacted with the primary antibody (as the "specific" stain) to cells not contacted with the primary antibody (as the "negative" control). The appropriate negative control would have been the use of an isotype/subtype matched nonspecific immunoglobulin. Alternatively, a cell that does not express the specific analyte could be used as the control. As discussed above with regard to the Karkmann *et al.* publication, the "negative" control used in the Lollini publication does not validate the specificity of the staining.

8. I have also determined that the no-antibody "negative" control used in the Lollini publication provides 2- to 4-fold less fluorescent signal than the isotype/subtype matched nonspecific immunoglobulin negative control with the tyramide amplification procedure. In contrast, standard flow cytometric staining procedures do not exhibit this differential between the no-antibody negative control and the isotype/subtype matched nonspecific immunoglobulin negative control. Thus, the 10- to 15-fold enhancement asserted in the Lollini publication is at best a 2-7.5 fold enhancement. Thus, the conditions disclosed in the Lollini publication for tyramide staining of intracellular analytes (phosphate buffered saline with 1% bovine serum

albumin and 0.5% Tween-20) do not provide specific staining or a 10-fold enhancement of signal in comparison to standard flow cytometry methods when isotype/subtype matched nonspecific immunoglobulin is used as a negative control, due to nonspecific background obtained when using the disclosed conditions

9. For these reasons, it is my opinion that the Karkmann *et al.* and Lollini publications do not disclose any methods in which cells comprising an intracellular analyte of interest are specifically labeled, or in which the signal obtained is at least 10-fold greater than a signal obtainable by standard flow cytometry methods using isotype/subtype matched nonspecific immunoglobulin as a negative control, as required by the instant claims.

10. In comparison to the cited publications, the conditions described in the present application can provide at least a 10-fold enhancement of an antigen-specific signal, and as shown in Figure 4, as much as a 150-fold enhancement of signal. Any increase in signal that may be obtained is considered critical to those of skill in the art, as an increase in specific signal indicates an increase in the detection sensitivity of the flow cytometric assay. Because these methods are often used in diagnosis of human diseases, this increase in detection sensitivity can translate directly into improved diagnosis.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Capital Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

10/14/02
Date

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EDUCATION

AB	Biology	University of Chicago, 1974
PhD	Immunology	University of Chicago, 1979
MD	Medicine	University of Chicago, 1980

RESIDENCY Pathology, Washington University, 1980-1984

PROFESSIONAL AND/OR RESEARCH EXPERIENCE

Research Fellow	Washington University	1981-1984
Assistant Professor of Pathology	CWRU	1984-1991
Associate Professor of Pathology	CWRU	1991-2000
Professor of Pathology	CWRU	2000-
Director, Immunology Labs	University Hospitals of Cleveland	1984-2000

PROFESSIONAL SERVICE

National Institutes of Health	member, review committees on transplantation,AIDS, and virology
National Institutes of Health	chairperson, 1995, review committee on AIDS
American Cancer Society	member, 1988-96, research committee
American Cancer Society	chairperson, 1992-1996, research committee
CWRU Cancer Center	member, 1988-92, grant review committee
CWRU, Department of Medicine	member, 1989, Research AIDS Virologist Search
CWRU, Institute of Pathology	chairperson, 1992, Research Immunologists Search Committee
CWRU, School of Medicine	site immunologist, 1987-, AIDS Clinical Trials Group (ACTG)
National Institutes of Health	member, 1997, ACTG, lymphocyte dynamics focus group
The Journal of Immunology	associate editor, 1999-

PROFESSIONAL SOCIETIES

International Society of Analytical Cytology
American Association of Immunologists
American Society for Microbiology
Great Lakes International Imaging and Flow Cytometry Association

TEACHING

Biological Basis of Disease (Lecturer)
Immunology of Infectious Diseases (Lecturer)
Cell Surfaces and Matrices (Lecturer)

AWARDS

Lederer Foundation Fellow	1974-1980
Hartford Foundation Fellow	1986-1989

PUBLICATIONS

Original papers

Kohler, H, **D Kaplan**, D Strayer. Clonal depletion in neonatal tolerance. **Science** 186:643, 1974.

Kaplan, D, J Quintans. Alteration of clonal profile: I. Effect of sublethal irradiation on the responses to phosphorylcholine in BALB/c mice. **J Exp Med** 148:987, 1978.

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PATENTS

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D Kaplan. Methods for detecting an analyte of interest using tyramide coating technology. Filed: 1/12/99. Issued: 1/1/02.

D Kaplan. Use of tyramide coating and physical separation for isolating cells or particles of interest. Filed: 5/25/99. Issued: 8/28/01.

D Kaplan. Methods for detecting an analyte of interest using catalyzed reporter deposition of tyramide. Filed: 12/15/00.

The Potential Role of Flow Cytometry in the Diagnosis of Small Cell Carcinoma

Dennis Cornfield, MD; Zach Liu, MD; Wojciech Gorczyca, MD, PhD; James Weisberger, MD

o *Context.*—Virtually no information exists in the medical literature on the immunophenotyping of small cell carcinoma by flow cytometry. CD56, or neural cell adhesion molecule, is widely expressed by small cell carcinoma and easily measured by flow cytometry.

Objective.—To determine the potential usefulness of flow cytometry in the diagnosis of small cell carcinoma.

Design and Setting.—Retrospective data and archival material on 27 patients were obtained from community hospitals. Specimens (needle aspirations and tissue biopsies) from all patients demonstrated cytomorphologic and flow cytometric features consistent with small cell carcinoma. All measurements were performed at a large reference laboratory. Routine 3- and 4-color flow cytometry using a lymphoma antibody panel, including anti-CD56, was performed. Anti-cytokeratin antibody was also used in the last 12 cases. Immunohistochemical staining with a panel of conventional markers for neuroendocrine neoplasms was performed on available tissue for purposes of confirmation of small cell carcinoma.

Patients.—Twenty-seven patients whose tissue specimens showed a clearly defined population of $CD45^{-}CD56^{+}$ cells by flow cytometry and cytomorphologic features consistent with small cell carcinoma.

Interventions.—Needle aspiration (n = 3) and tissue biopsy (n = 24) from a variety of sites.

Results.—CD56 positivity by flow cytometry was 100 to 1000 times that of the matched isotype control in 25 cases and 10 to 100 times that of the control in 2 cases. Cytokeratin positivity by flow cytometry was found in 12 of 12 cases. Immunohistochemical staining showed positivity for at least 1 cytokeratin and 1 or more neuroendocrine markers in 26 of 27 cases and confirmed the diagnosis of small cell carcinoma.

Conclusions.—Routine flow cytometry can identify a neuroendocrine phenotype that shows a strong correlation with confirmatory immunohistochemical markers in cases exhibiting cytomorphologic features of small cell carcinoma. Flow cytometry appears to complement and may possibly be a satisfactory alternative to immunohistochemical staining when small cell carcinoma is suspected.

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The diagnosis of small cell carcinoma (SCC) is typically based on a combination of characteristic morphology and neuroendocrine phenotype as demonstrated by immunohistochemical (IHC) staining. Among the neuroendocrine markers consistently expressed by SCC is the neural cell adhesion molecule, a member of the immunoglobulin superfamily of adhesion molecules, which play a role in cell-cell adhesion in normal and malignant tissues, especially in tumors with neuroendocrine differentiation.[1,2] Neural cell adhesion molecule can exist in several isoforms, one of which, the 140-kd isoform, has been shown to be identical to the human leukocyte differentiation antigen CD56.[3]

Small cell carcinoma is usually densely cellular and easily disaggregated by mechanical means. These features, together with expression of CD56 in virtually all cases, make SCC an excellent candidate for phenotypic analysis by flow cytometry (FCM).

Although the medical literature contains numerous articles on FCM analysis of the DNA content of SCC,[4–6] there is virtually no information available on the contribution of FCM-determined immunophenotyping to the diagnosis of SCC.[7] From specimens submitted to IMPATH, Inc (New York, NY) for FCM in the last 2 years, we identified 38 cases whose FCM characteristics and morphologic features suggested a diagnosis of SCC. Where sufficient tissue was available (27 cases), we applied a panel of conventional IHC markers used to identify neuroendocrine neoplasms. The results of FCM and IHC were compared, with attention to CD56 expression. We sought to determine the potential diagnostic usefulness of FCM in SCC, based on these 27 cases.

MATERIALS AND METHODS

Case Material

Twenty-seven cases were selected for IHC staining based on FCM demonstration of a clearly defined population of $CD56^{+}CD45^{-}$ cells along with cytologic/morphologic features of SCC, namely, cohesive, small to medium-sized cells with condensed stippled nuclear chromatin, inapparent nucleoli, nuclear molding, and often necrosis and streaming of nuclear chromatin. Tissue from the following sites was evaluated: intrathoracic, including mediastinum (n = 5) and lung (n = 1); peripheral lymph nodes (n = 15); bone marrow (n = 1); liver (n = 3); submandibular gland (n = 1); and nasal cavity (n = 1). Three cases represented tissue aspirates (1 bone marrow, 2 liver); the remaining 24 cases consisted of tissue biopsies.

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Results Obtained by Flow Cytometry and Immunohistochemistry (27 Cases)*									
Case No.	Flow Cytometry		Immunohistochemistry						
	CD56	Cytokeratin	CD56	CAM5.2	TTF-1	AE1/AE3	Chromogranin	Synaptophysin	NSE
1	Moderate to bright	...	+	+	...	...	+	+	+
2	Bright	Dim	...	...	−	Indeterminate	−	+	+
3	Bright	...	+	+	+	+	+	+	+
4	Bright	...	+	+	+	+	+	+	+
5	Variable	...	+	+	+	+	+	+	+
6	Moderate to bright	...	+	+	+	+	+	+	+
7	Moderate to bright	...	+	+	−	+	+	+	+
8	Bright	...	+	+	−	+	−	+	+
9	Bright	...	+	+	+	+	+	+	+
10	Bright	...	+	+	−	+	−	+	+
11	Dim to moderate	...	+	+	−	+	−	+	+
12	Bright	...	...	+	−	...	+	+	+
13	Bright	...	+	+	+	+	+	+	+
14	Bright	...	+	...	−	+	−	...	+
15	Bright	...	+	+	+	+	+	+	+
16	Bright	...	+	+	+	+	+	−	+
17	Bright	Moderate	+	+	+	...	+	+	+
18	Bright	Moderate	+	+	+	+	+	+	+
19	Moderate to bright	Moderate	+	+	+	+	+	+	+
20	Bright	Bright	+	+	+	+	+	+	+
21	Bright	Moderate	+	+	+	+	+	+	+
22	Bright	Moderate	+	+	+	+	+	−	+
23	Bright	Moderate	+	+	−	+	+	+	+
24	Bright	Moderate	+	+	+	+	−	+	+
25	Bright	Moderate	+	+	+	+	+	+	...
26	Bright	Moderate	+	...	...	+	+	+	...
27	Bright	Moderate	+	+	...	+	−	...	Indeterminate

* TTF-1 indicates thyroid transcription factor-1; NSE, neuron-specific enolase. Dim, moderate, and bright indicate intensity of expression was approximately 10, 100, or 1000 times, respectively, that of the matched isotype control. Ellipses indicate test was not performed.

Flow Cytometry

Immunophenotyping by standard 3- or 4-color FCM was performed in accordance with guidelines outlined in the 1995 US-Canadian consensus conference on FCM.[8] Briefly, cell suspensions were prepared from solid tissues using a manual dispersion method. Isolated cells were preincubated in RPMI 1640 medium supplemented with 10% fetal bovine serum to minimize nonspecific binding of antibodies. Erythrocytes in bone marrow specimens were lysed with a 0.008% solution of ammonium chloride. Cells were washed with phosphate-buffered saline and incubated with Becton Dickinson cocktails of antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, or allophycocyanin. The antibodies chosen comprised a panel used by IMPATH, Inc, to evaluate and characterize lymphoproliferative disorders. The last 12 cases also included a fluorescein isothiocyanate–conjugated antibody to cytoplasmic cytokeratin (Dako Corporation, Carpinteria, Calif). Data acquisition and analysis were performed on a FACScalibur FCM instrument. CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif) was used for data analysis. Antigen expression as assessed by fluorescence intensity was depicted on multiple dual-parameter scattergrams. Nonviable cells were excluded by using 7-amino actinomycin D (7-AAD). Matched isotype controls were used in all FCM panels. Cells were considered positive for antigen expression if they were present in a well-defined population whose median fluorescence intensity was approximately 1 or more logs greater than that of its matched isotype control.

Immunohistochemistry

Tissue from 27 cases was available for immunostaining. After deparaffinization and standard antigen retrieval, immunostaining was performed on 4-μm tissue sections using the TechMate 500 automated immunostainer (Ventana Medical Systems, Tucson, Ariz) and the EnVision detection system (Dako). The following antibodies were used (name, dilution, and manufacturer): CAM5.2, 1:200, Becton Dickinson; AE1/AE3, 1:100, Dako; synaptophysin, 1:300, Dako; chromogranin, 1:16 000, Dako; neuron-specific enolase, 1:4000, Dako or Chemicon International, Inc, Temecula, Calif; CD56, 1:100, Neomarkers, Inc, Fremont, Calif; thyroid transcription factor-1 (TTF-1), 1:1000, Neomarkers.

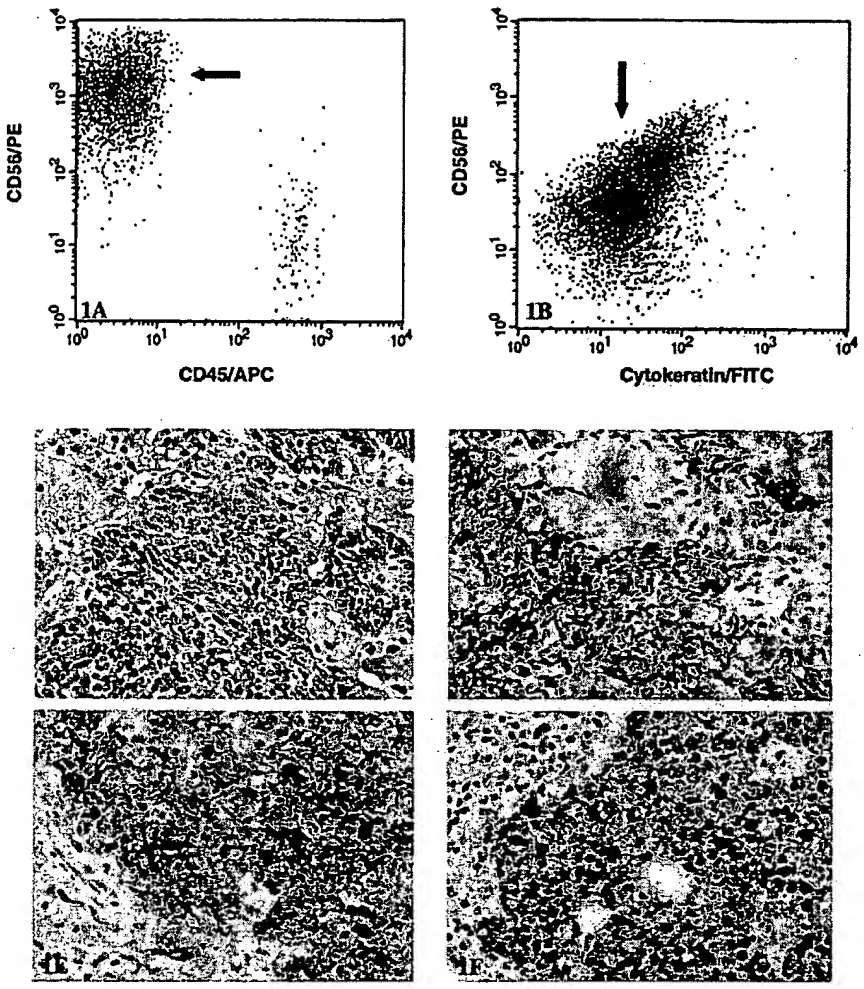
RESULTS

Flow Cytometry

The Table summarizes the pertinent flow cytometric and immunohistochemical findings. The SCC cell size, as determined by forward scatter, was always large, with only mild internal complexity (side scatter). The intensity of CD56 expression was moderate to bright (approximately 100–1000 times that of the matched isotype control) in 25 cases and dim to moderate (10–100 times that of the control) in 2 cases (Figure, A). CD38 expression was negative in all cases, arguing against a plasma cell neoplasm. Cytokeratin testing was performed in 12 cases; all exhibited positivity (1 dim, 10 moderate, 1 bright), confirming the epithelial nature of the nonhematopoietic neoplastic population (Figure, B).

Immunohistochemistry

Twenty-six of 27 cases in which tissue was available for IHC staining exhibited a profile consistent with a carcinoma of neuroendocrine differentiation, that is, positivity for cytokeratin and for 1 or more conventional neuroendocrine markers. (Although the AE1/AE3 stain yielded an indeterminate result in case 2, the case was felt to be an SCC based on cytomorphologic features and positivity for synaptophysin and neuron-specific enolase.) The results were as follows (No. positive/No. studied): AE1/AE3, 22/23; CAM5.2, 24/25; TTF-1, 16/24; CD56, 25/25; chromogranin, 20/27; synaptophysin, 23/25; and neuron-specific



Typical case of small cell carcinoma. Scattergrams show the population of malignant cells to be CD56⁺CD45⁻ (A) and cytokeratin⁺ (B). The arrows denote the malignant population. Cells have condensed stippled nuclear chromatin and exhibit nuclear molding (C) (hematoxylin-eosin, original magnification ×200). Immunostains show positivity for AE1/AE3 (D), CD56 (E), and neuron-specific enolase (F) (all original magnifications ×200).

enolase, 24/25. Thyroid transcription factor-1 is a transcription factor expressed in epithelial cells of lung and thyroid origin and is usually negative in cells of gastrointestinal, genitourinary, breast, and skin origin. Positivity of TTF-1 in SCC is presumptive evidence of pulmonary origin, given the rarity of SCC of the thyroid.

COMMENT

In this group of patients, who were selected because of an FCM-determined CD56⁺CD45⁻ phenotype and cytomorphologic features of SCC, we found very high concordance with results obtained using a panel of conventional IHC markers for neuroendocrine neoplasms. These results suggest that FCM complements and may possibly be a satisfactory alternative to IHC when SCC is suspected.

Flow cytometry has a number of advantages over IHC. Intensity of antigen expression is quantifiable and can be expressed in objective numeric terms. In many cases, FCM can be performed on material obtained by fine-needle aspiration. This feature, together with the rapidity with which results can be available (often within hours), makes FCM a potentially valuable tool for situations in which more invasive procedures can be hazardous and a speedy diagnosis is desirable, for example, in patients symptomatic from superior vena cava syndrome. When aspirate samples are too scanty or are otherwise inadequate for FCM, IHC would continue to serve as the standard confirmatory modality for diagnosing SCC.

Positivity for the cytokeratin antibody, as demonstrated in all 12 cases in which it was tested by FCM, suggests that it may be a useful addition to a standard lymphoma antibody panel when a nonhematopoietic neoplasm such as SCC is in the differential diagnosis. Of possible interest, in the 11 cases not included in this series because of lack of additional tissue for IHC confirmation, 7 of 7 cases evaluated by FCM showed cytokeratin positivity.

A common problem for surgical pathologists is differentiation of SCC or other small blue cell tumors from malignant lymphoma because of overlapping cytomorphologic features. In our series, standard FCM graphic display of CD45 plotted against CD56 typically demonstrated the population of interest to be clearly separate from lymphoid and other hematopoietic cells (Figure, A), thereby providing a distinction from most lymphomas, as well as suggesting a neoplasm of neuroendocrine derivation. The only medical literature we could find that alluded to FCM phenotyping in the diagnosis of SCC was a recent abstract by investigators at the University of Washington.[7] Eleven of 16 cases of small blue round cell tumors exhibiting strong expression of CD56 and negativity for CD45 by FCM were diagnosed as SCC, including 1 case with very low-level bone marrow involvement.

We emphasize that our series was selected based on the $CD56^+CD45^-$ phenotype, together with morphologic features consistent with SCC. We did not attempt to assess the sensitivity or specificity of this phenotype. CD56 is not unique to neural/neuroendocrine tumors and can be expressed, to some extent, by a wide variety of malignancies, including cancer of the gallbladder,[9] malignant mesothelioma,[10] and non–small cell cancer of the lung.[11] In a study of 889 cases of non–small cell lung cancer, 86 (15%) of 575 cases of squamous cell carcinoma and 30 (11%) of 262 cases of adenocarcinoma demonstrated IHC positivity for neural cell adhesion molecule,[11] findings which underscore the importance of careful correlation of cytomorphology with immunophenotypic findings. Plasma cell neoplasms are often $CD56^+$ and $CD45^-$, but they usually express CD38, unlike SCC, and their morphologic differentiation from SCC is generally straightforward. In rare cases of pleural effusion, a cell population with the immunophenotypic profile of SCC has been noted, but only benign mesothelial cells were seen on the cytospin preparation (J.W., unpublished data, April 2002).

The present study is retrospective and reflects an element of case bias. Material was submitted for FCM because lymphoma was suspected in many instances. Twenty of 27 specimens were lymph nodes, either mediastinal or extrathoracic. It is unclear whether the same results would have been obtained from an unselected series of patients with SCC undergoing fine-needle aspiration of lesions from a wider variety of sites. Despite these limitations, our results appear to indicate that the combination of the $CD56^+CD45^-$ phenotype, as determined by FCM, and typical small cell neuroendocrine-type morphology, in the proper clinical setting, is highly suggestive of SCC. Immunophenotyping by routine 3- or 4-color FCM is relatively simple, rapid, and efficient and provides information that can be useful clinically. Confirmatory studies on larger numbers of patients will be necessary to define the possible role of FCM in the approach to the diagnosis of SCC.

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CD38 ligation inhibits normal and leukemic myelopoiesis

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CD38 is a transmembrane molecule whose expression varies during hematopoietic cell differentiation. We used stroma-supported cultures of human myeloid cells to assess the effects of CD38 ligation on myeloid differentiation. In 8 experiments with CD34+ cells purified from normal bone marrow or cord blood, flow cytometry used with antibodies to CD34 and myeloperoxidase (MPO) identified 4 cell populations after 7 days of culture. Addition of anti-CD38 (T16) to the cultures induced a profound reduction of the most mature (CD34−MPO++) cell population, which includes promyelocytes, myelocytes and metamyelocytes; mean (± SD) cell recovery was 12.8% ± 9.8% of that in parallel cultures with an isotype-matched control antibody. The suppressive effect of CD38 ligation on phenotypically more immature normal cells was inconsistent but generally less pronounced. Recovery of CD34++MPO− cells was 63.3% ± 24.4%, recovery of CD34[+/−] MPO− cells was 95.3% ± 35.1%, and recovery of CD34−MPO+ cells was 42.0% ± 18.7% of that in control cultures. However, anti-CD38 suppressed recovery of cells obtained from 6 patients with CD38+ acute myeloid leukemia; after 7-day cultures, cell recovery was 25.2% ± 21.7% of that in control cultures. Cell recovery was also reduced by F(ab')2 or Fab fragments of anti-CD38. CD38 ligation dramatically suppressed recovery of murine 32D myeloid cells transfected with human CD38 and cocultured with stroma (3.8% ± 7.3%; n = 7). CD38 ligation of CD38+ 32D cells also induced cell aggregation, tyrosine kinase activity, and Ca++ influx. We conclude that CD38 mediates signals that culminate in suppression of myeloid cell growth and survival. (Blood. 2000;95:535-542)

Introduction

CD38 is a 45-kDa transmembrane molecule that is expressed heterogeneously during lymphohematopoietic cell differentiation. Most human immature hematopoietic cells with high potential for self-renewal and multilineage differentiation express low levels of CD38 or no detectable CD38 at all.[1-6] Conversely, lineage-committed myeloid and lymphoid progenitor cells express very high levels of CD38, which then decreases dramatically as maturation progresses. In the lymphoid lineage, CD38 is again expressed intensely by activated lymphocytes and plasma cells.[7-10]

It is not yet clear whether such remarkable changes in CD38 expression simply reflect cell cycle and activation status or whether CD38 participates in the regulation of cell growth and differentiation at certain maturation stages. The latter possibility is supported by the fact that in leukemic immature cell lines, ligation of CD38 with specific antibodies results in a rapid and transient increase in cellular tyrosine kinase activity and in phosphatidyl inositol 3-kinase activity associated with the transmembrane molecule CD19 and the adaptor molecule CBL.[11-18] CD38 ligation also induces growth arrest and apoptosis in normal and leukemic immature B lymphoid cells cultured on bone marrow-derived stromal layers,[10] supporting the premise that CD38 plays a functional role in lymphohematopoiesis.

The cellular effects mediated by ligation of CD38 in normal immature myeloid cells, which express high levels of CD38,[19,20] have not yet been thoroughly elucidated. In this study, we found that CD38 ligation transduced signals that virtually abrogated the myeloid cell differentiation of normal CD34+ cells in coculture with bone marrow stroma. The suppressive effect mediated by CD38 was also observed in experiments with patient-derived myeloid leukemic cells and with the murine cell line 32D transfected with human CD38 cDNA.

Materials and methods

Cells and antibodies

Cord blood samples were obtained after normal full-term deliveries. Bone marrow samples were taken from 6 healthy bone marrow transplant donors aged 9 to 33 years (median, 20 years) and from 7 patients aged 7 to 15 years (median, 11 years) with newly diagnosed acute myeloid leukemia (AML). The diagnosis was unequivocal by morphologic, cytochemical, and immunophenotypic criteria. These studies were approved by the Institutional Review Board, with informed consent obtained from patients or their parents or guardians. Mononucleated cells were separated on a density gradient (Lymphoprep; Nycomed, Oslo, Norway) and washed 3 times in RPMI-1640 (BioWhittaker, Walkersville, MD). Normal CD34+ cells were separated by using a MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), which consistently affords a purity of 90% or greater. The interleukin-3 (IL-3)-dependent murine immature myeloid cells 32D cl3 (32D)[21] were available in our laboratory. They were cultured in RPMI-1640 supplemented with IL-3 (25 U/mL; derived from CHO cells expressing the murine IL-3 gene), 10% fetal calf serum (FCS; BioWhittaker), L-glutamine, and antibiotics. Bone marrow-derived stromal layers

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were prepared in flat-bottomed 96-well plates (Costar, Cambridge, MA) and fed with RPMI-1640, 10% FCS, and 10^{-6} mol/L hydrocortisone (Sigma, St. Louis, MO), as previously described.[10,22-26] Monoclonal anti-CD38 antibodies were T16 (IgG1; Immunotech, Westbrook, ME) and THB7 (IgG1; American Type Culture Collection [ATCC], Rockville, MD). Fab and $F(ab')_2$ fragments of THB7 (prepared in one of our laboratories) were used in some experiments. The purity of the latter reagents was verified by sodium dodecyl sulfate–polyacrylaminde gel electrophoresis (SDS-PAGE).

DNA constructs and electroporation conditions

The CD38 expression vector was constructed by excising the human CD38 cDNA fragment from pCDM:CD38 (a gift from Dr D. G. Jackson, Oxford, UK) with *Xba*I and inserting it into an *Xba*I-cleaved pEF-BOS mammalian expression vector (a gift from Dr S. Nagata, Osaka, Japan).[27] Either the expression plasmid (32 μg) or the pEF-BOS vector without insert was electroporated into 5×10^6 32D cells with 3.2 μg of a second plasmid (pSTneoB) by using a gene pulser apparatus (Bio-Rad, Richmond, CA) set at 960 μF and 290 V. Cells were cultured for 24 hours in RPMI-1640 plus additives (see above). Transfected cells were selected after culture in the presence of 1 mg/mL G418 (Life Technologies, Gaithersburg, MD). Individual clones were obtained by single-cell sorting using a FACS Vantage flow cytometer equipped with an automatic cell deposition unit (Becton Dickinson, San Jose, CA). Clones were expanded and screened for cell surface expression of human CD38 by labeling with anti-CD38 conjugated to fluorescein isothiocyanate (FITC).

Cell culture studies

Before each experiment, we washed the adherent stromal cells with tissue culture medium. Normal $CD34^+$ cells and leukemic myeloblasts were resuspended in AIM-V serum-free medium (Life Technologies). The 32D cells transfected with cDNA encoding the human CD38 or with the vector only were resuspended in fresh RPMI-1640 with all the additives, including IL-3 (see above). Two hundred microliters of each cell suspension was then seeded onto marrow stromal cells. In each well, we placed 0.5 to 1×10^5 normal $CD34^+$ cells, 2×10^5 AML cells, and 0.2 to 0.5×10^5 32D cells. In some experiments, transfected 32D cells were placed into the empty wells of a 96-well flat-bottomed microtiter plate or into the wells of Transwell culture supports with 0.4-μm microporous membrane inserts (Corning Costar, Cambridge, MA). For culture experiments, anti-CD38 antibodies and nonreactive control Ig were dialyzed in phosphate-buffered saline (PBS), sterile-filtered, and used at concentrations of 2 to 10 μg/mL. All cell cultures were incubated at 37°C in 5% CO_2 with 90% humidity. Stromal layers remained adherent throughout the cultures.

Cell counting and assessment of ploidy and apoptosis

After culture, cells were harvested by pipetting, suspended in PBS, and passed through a 19-gauge needle to disrupt clumps. Viable cells were enumerated by flow cytometry, as previously described.[10,22-26] Normal myeloid cells were stained with anti-CD34 conjugated to peridin chlorophyll protein (PerCP) or phycoerythrin (PE; both from Becton Dickinson), anti-CD14 conjugated to PE (Becton Dickinson), and anti-myeloperoxidase (MPO) conjugated to FITC (Dako, Carpinteria, CA). The latter was added to the cells after cell permeabilization with Fix & Perm (Caltag; Burlingame, CA).[28] Anti-CD13 PE or FITC, anti-CD33 FITC, anti-glycophorin A FITC (all from Dako), anti-CD38 FITC and/or CD19 PE (both from Becton Dickinson) were also used to label normal and leukemic myeloid cells. Cell cycle analysis was done as previously described,[29] using the ModFit software (Becton Dickinson). To detect apoptosis, we labeled phosphatidylserine residues exposed on the cell surface with FITC-conjugated Annexin-V (Trevigen, Gaithersburg, MD), following the manufacturer's instructions.[30] In these experiments, cell membrane permeabilization was revealed by labeling cells with 5 μg/mL propidium iodide (Trevigen) for 15 minutes at 20°C.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed essentially as previously described.[11,12,16,18,27] Briefly, after exposure to anti-CD38 antibody or control Ig (5-10 μg/mL), 32D cells were lysed in 1 mL of lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% [v/v] Triton X-100, 5 μg/mL aprotinin, 1 mM phenylmethyl sulfonyl fluoride, 1 mM EDTA, and 1 mM Na_3VO_4). After centrifugation at 20,000*g* for 20 minutes, cell lysates were diluted with sample buffer (10% [v/v] glycerol, 5% 2-mercaptoethanol, 3% [w/v] SDS, 65 mM Tris-HCl [pH 6.8], and 0.002% [w/v] bromophenol blue) and separated on a 7.5% acrylamide gel.[11,12,16,18] After transfer, nitrocellulose filters were incubated first in 5% albumin in TBS-T20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween20 for 12 hours, and then with the antiphosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) for 1 hour. The filters were washed in TBS-T and incubated for 1 hour with horseradish peroxidase-conjugated sheep anti-mouse Ig (Amersham Corp., Arlington Heights, IL). The filters were then washed, incubated with enhanced chemiluminescence detection reagents (Amersham), and exposed to Kodak BioMax MR film.

Measurement of Ca^{++} flux

The 32D cells were resuspended in RPMI-1640 at a concentration of 1×10^6/mL. The Ca^{++}-binding fluorochrome Indo-1 (Molecular Probes, Eugene, OR) was then added at a final concentration of 10 μM and the suspension was incubated at 20°C for 30 minutes. Ca^{++} flux was measured by using a fluorescence-activated cell sorter (FACS) Vantage flow cytometer equipped with an ILT argon-ion laser tuned to deliver 50 mW at 488, and a Coherent 306 laser tuned to deliver multiline UV light at 80 mW, exciting Indo-1 fluorescence. After splitting with a 440 LP dichroic filter, the incident fluorescence emission was measured by 2 detectors at wavelengths of 400 ± 40 nm and 480 ± 40 nm. The ratio of the 2 linear fluorescence signals was used as the indicator of Ca^{++} flux.

Results

Effects of CD38 ligation in cultures of normal $CD34^+$ cells

To investigate the effects of CD38 ligation on the growth of normal myeloid progenitors, we separated $CD34^+$ cells (> 90% of which are $CD38^+$) from cord blood and normal bone marrow samples by using magnetic beads conjugated to anti-CD34 (Figure 1). We then seeded the cells onto allogeneic bone marrow stromal layers, which in preliminary experiments supported their survival and proliferation as well as their differentiation to mature myeloid cells (unpublished observations). After the culture period, we used flow cytometry to quantify the effects of CD38 ligation on normal myeloid cell differentiation. The cells were stained simultaneously with antibodies to CD34 and to MPO, permitting identification of 4 distinct cell populations (Figure 2). The most immature cells (populations 1 and 2) had very low or undetectable MPO expression; population 1 had high levels of CD34 expression, whereas population 2 was $CD34^{dim}$ or $CD34^-$. The most differentiated cells (populations 3 and 4) expressed MPO; cells in population 3 had intermediate levels of MPO and were $CD34^{dim}$ or $CD34^-$, whereas those in population 4 had high levels of MPO and were invariably $CD34^-$. Cells in all 4 populations expressed CD13 and/or CD33 (myeloid markers), and lacked CD19 and glycophorin A (B cell and erythroid markers, respectively), indicating their myeloid association (not shown). Population 4 includes cells that have differentiated to the promyelocyte stage or further, whereas the remaining populations include more immature myeloid cells; monocytes constitute a proportion of population 3.[28,31,32]

The number of cells recovered after 7 days of culture with a nonreactive antibody ranged from 142% to 1230% (median, 295%)

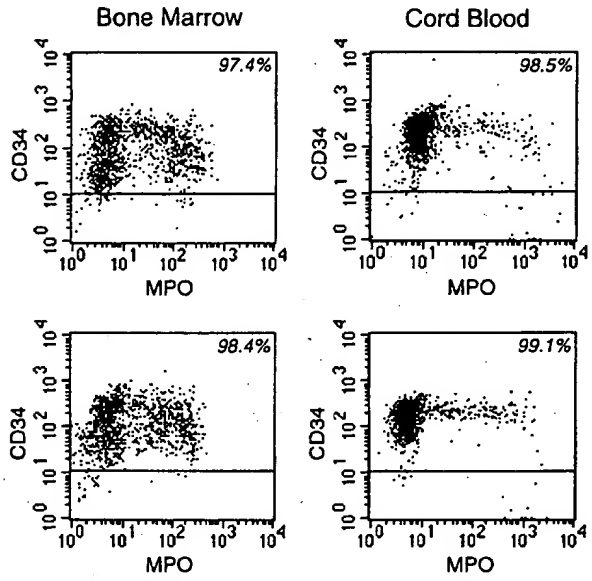

Figure 1. CD34 and MPO expression of enriched normal CD34+ cells from 2 bone marrow and 2 cord blood samples before culture. The percentage of CD34+ cells in each experiment is indicated.

of those originally seeded. Mean (± SD) cell recovery in the presence of anti-CD38 was consistently lower (49.7% ± 21.6%; n = 8) than that in control cultures with the nonreactive antibody (isotype-matched). The reduction in cell numbers, however, differed markedly between cell populations at different stages of maturation (see Figure 2). Cell recovery was particularly low among the most mature "population 4" cells (12.8% ± 9.8% of cell recovery in control wells). In line with these results, virtually no promyelocytes, myelocytes, or metamyelocytes could be identified in Wright-Giemsa-stained cytocentrifuge preparations from cultures containing anti-CD38 (not shown). By contrast, the effect of CD38 ligation on the most immature population 1 and population 2 cells was inconsistent and, overall, cell recovery was significantly less affected (63.3% ± 24.4% and 95.3% ± 35.1% of control cultures, respectively; $P < 0.001$ by t test for both comparisons).

Cell recovery of the intermediate population 3, which includes both granulocytic and monocytic cells,[31,32] was 42.0% ± 18.7%. To determine whether CD38 ligation affected the development of both cell lineages or was selective for 1 lineage, we labeled cells at the end of the cultures with anti-CD14, a marker expressed by monocytic but not granulocytic cells. In 4 experiments (2 with cord blood and 2 with bone marrow CD34+ cells), the number of MPO+CD14+ cells at the end of 7 days of culture in the presence of anti-CD38 was 55.4%, 63.9%, 46.9%, and 15.0% of that in control cultures. In another experiment with bone marrow-derived CD34+ cells, the cultures were prolonged to 13 days; after culture in the presence of anti-CD38, the number of MPO+CD14+ cells was 18.8% of control culture values. These results indicate that both granulocytic and monocytic cells are sensitive to CD38-mediated inhibition.

Effects of CD38 ligation in cultures of patient-derived leukemic myeloid cells

The inhibitory effects of CD38 ligation were also seen in stroma-supported cultures of leukemic myeloid cells. Expression of CD38 was heterogeneous among cells derived from 6 patients with AML; cells from 1 additional patient were CD38−. Table 1 summarizes the main presenting clinical and cellular features of the CD38+ cases. Percentage of cell recovery after 7 days on allogeneic bone marrow stroma ranged from 68% to 230% (median, 121%) of the viable cell input. When anti-CD38 (T16) was added to the cultures, cell recovery was decreased in all 6 CD38+ cases (mean cell recovery = 25.2% ± 21.7% of control cultures with isotype-matched nonreactive antibody; see Table 1; Figure 3). A similar decrease in cell numbers, albeit less marked (46.0% ± 16.8% of recovery in control cultures), was seen in parallel cultures with the anti-CD38 antibody THB7. By contrast, in the patient whose leukemic cells did not express CD38, neither anti-CD38 antibody significantly affected cell recovery. The extent of cell recovery inhibition did not directly correlate with intensity of CD38 expression (see Table 1). On the contrary, the 2 patients with the lowest cell recovery (patients 1 and 3) expressed CD38 at the lowest level in this series, whereas the patient with highest cell recovery (patient 5) had the highest level of CD38 expression. CD38-mediated inhibition of cell recovery appeared to be caused at least in part by induction of apoptosis, as indicated by cells with

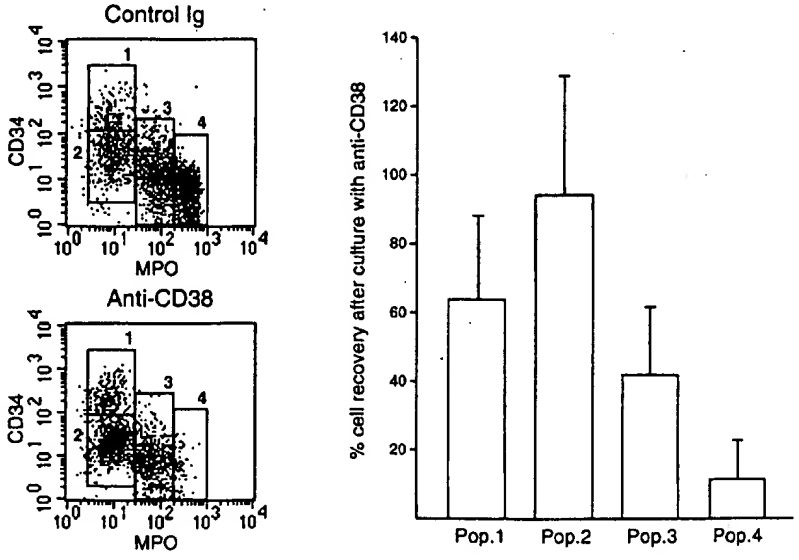

Figure 2. CD38 ligation induces a block in normal myeloid cell differentiation. CD34+ cells were cultured for 7 days on allogeneic bone marrow stromal layers in the presence of anti-CD38 (T16) or of a control nonreactive Ig. After culture, 4 phenotypically distinct cell populations could be identified by staining with anti-CD34 PE and anti-MPO FITC (left panels). Right panel shows percent cell recovery of each of the 4 subpopulations in the presence of anti-CD38 as compared with parallel control cultures with nonreactive Ig; results are mean (± SD) of 8 experiments.

Table 1. Presenting features of patients with CD38+ AML and responses to culture with anti-CD38

Pt	Age (y)	WBC ($\times 10^9$/L)	FAB	Karyotype	CD38 Expression* (MFI)	Cell Recovery After Culture With Anti-CD38†
1	11	98.4	M2	46,XX,t(6;9)(p23;q34) [31]	32.2	4%
2	7	9.9	M4	46,XX,inv(16)(p13.1q22) [10]/46,XX [12] 47,XX,der(2)t(1;2)(q21;q35),t(5;12)	82.3	36%
3	14	26.2	M1	(p13;p13),del(8)(p21),+22 [19]/46,XX [1]	17.7	8%
4	7	88.8	M2	46,XY [16]	61.1	17%
5	10	69.3	M1	46,XY [40]	94.6	63%
6	15	86.2	M5	48,XY,+8,+13 [4]/46,XY [19]	57.3	23%

WBC, white blood cell count; FAB, French-American-British classification; MFI, mean fluorescence intensity.

*Percentage of cells with CD38 staining above the highest levels achievable with an isotype-matched control antibody was >90% in patients 2, 4, 5, and 6, 75% in patient 1 and 44% in patient 4. In the two latter patients, however, the whole cell population was shifted and no distinct CD38 negative subset was detectable.

†Cell recovery after 7 days of culture on allogeneic bone marrow stroma in the presence of anti-CD38 (T16) is expressed as a percentage of cells recovered after parallel cultures with an isotype-matched nonreactive antibody. Results are the mean of 4 measurements.

nuclear fragmentation in cultures containing anti-CD38 (not shown) and by characteristic shifts in light scattering seen in some cases (eg, patient 3 in Figure 3) at the end of the cultures. After 48 hours of culture with anti-CD38, there was a marked increase in Annexin V labeling and hypodiploidy (Figure 4), hallmarks of apoptosis.[33]

Inoue et al[17] have proposed that CD38-mediated tyrosine phosphorylation in myeloid cell lines requires simultaneous engagement of CD38 and FcγRII receptors. To determine whether Fc receptor signaling was required for the cellular effects caused by CD38 ligation in leukemic myeloid cells, we performed parallel cultures to which $F(ab')_2$ fragments of THB7 were added. This reagent suppressed cell recovery (58.0% ± 14.6% of that in control cultures) in the 6 CD38+ AML cases. Thus, Fc receptor signaling is not required for CD38-mediated suppression of cell growth in leukemic myeloblasts. Leukemic cell recovery was also reduced (52.2% ± 27.0% of control) in cultures to which a Fab monomeric fragment of THB7 was added. Thus, conformational changes in the CD38 molecule, rather than cross-linking, appear to be important for CD38-mediated suppression of cell growth.

Effects of CD38 ligation on 32D cells transfected with human CD38

To further investigate the effects of CD38 ligation in myeloid cells, we transfected the murine cell line 32D (which does not express CD38; unpublished observation) with a human CD38 cDNA. Clonal 32D cells expressing high levels of human CD38 were obtained after transfection and single-cell sorting. In the selected clones, cell-surface CD38 expression was similar to or greater than that of human normal and leukemic immature myeloid cells. By contrast, mock-transfected 32D cells did not react with anti-human CD38 antibodies.

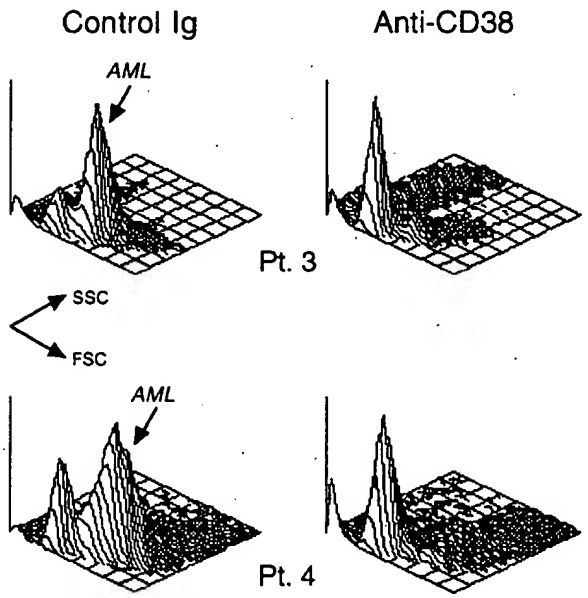

Figure 3. CD38 ligation inhibits in vitro growth of patient-derived leukemic myeloid cells. AML blast cells from 2 patients (patients 3 and 4 in Table 1) were cultured for 7 days on allogeneic bone marrow stromal layers in the presence of anti-CD38 (T16) or of a control nonreactive Ig. Isometric contour plots depict the cells' light scattering (FSC, forward scatter; SSC, side scatter) after culture. In cultures with control Ig, most cells had light-scattering properties of viable myeloblasts. These cells also expressed CD13 and/or CD33 (not shown). Myeloblast cell recovery was drastically reduced in cultures with anti-CD38, where most residual viable cells had lymphoid morphology, and lacked CD13 and CD33 (not shown).

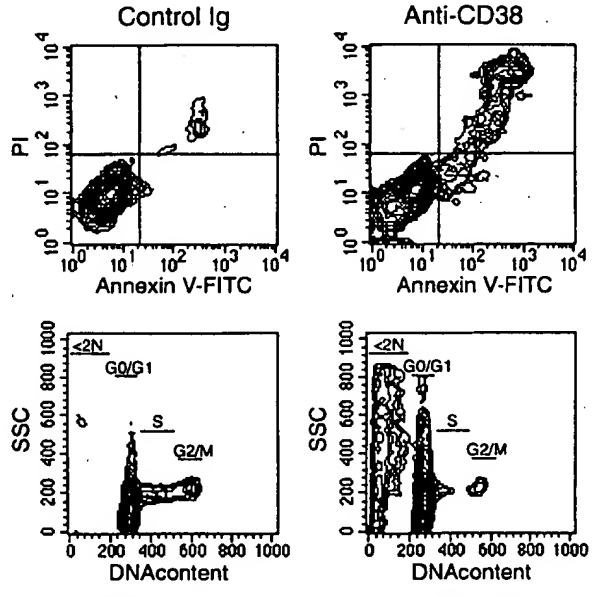

Figure 4. Ligation of CD38 induces apoptosis in AML cells. Leukemic myeloblasts (patient 3 in Table 1) were cultured for 48 hours on allogeneic bone marrow stromal layers in the presence of anti-CD38 (T16) or of a control nonreactive Ig. Top panels are flow cytometric contour plots illustrating staining with Annexin-V (x-axis; a marker of apoptosis) and propidium iodide (PI, y-axis; a marker of cell membrane permeability) after culture. Bottom panels illustrate DNA content analysis; a marked increase in hypodiploid (< 2N) cells, characteristic of apoptosis, is seen in cultures containing anti-CD38. Among the viable cells, the percentage of cells in G_0/G_1, S, and G_2/M was 90%, 8%, and 2% with control Ig, and 95%, 4%, and 1% with anti-CD38, respectively.

Ligation of human CD38 with T16 or THB7 induced aggregation of 32D cells transfected with CD38 (Figure 5). This effect was seen in all 4 $CD38^+$ clones studied but not in cells transfected with vector only or in human myeloid cells. Cell aggregation resembled that observed with Ba/F3 murine pro-B cells transfected with human CD38.[27] Aggregation became distinguishable within 2 hours of exposure to the antibody and was maximal after 24 hours. Aggregation was also induced by $F(ab')_2$ and Fab fragments of THB7 (see Figure 5). As we previously observed with Ba/F3 murine pro-B cells tranfected with human CD38,[27] cell aggregation was not noticeably reduced by the addition of 5 mM EDTA or 5 mM EGTA to the cultures, indicating that aggregation did not require Ca^{++} or Mg^{++}. Neither was aggregation inhibited by preincubating cells for 90 minutes with an antibody to leukocyte function-associated antigen 1 (LFA-1) (I21/7.7), indicating that the interaction between LFA-1/ICAM-1 was not involved.

When CD38-transfected 32D cells (3 different clones) were cultured with anti-CD38 (T16), cell recovery after 7 days varied but was slightly less overall than that in control wells that contained a control nonreactive antibody; the mean (± SD) cell recovery in 15 experiments was 77.1% ± 23.1% (Figure 6). However, when bone marrow-derived stroma was present in culture wells, cell recovery in the presence of anti-CD38 antibody (T16) decreased dramatically (3.8% ± 7.3%; n = 7; see Figure 6). Similar results were seen with THB7 and with the $F(ab')_2$ and Fab fragments of this antibody. In a comparative experiment, mean cell recovery of duplicate cultures relative to control cultures with isotype-matched nonreactive Ig was 2.7% with T16, 3.7% with THB7 whole Ig, 2.7% with THB7 $F(ab')_2$, and 3.2% with THB7 Fab. No decrease in cell recovery was observed in parallel experiments with mock-transfected 32D cells. These results recall our observations that in human immature B-cell lines CD38 ligation induces growth suppression only when cells are cultured in the presence of bone marrow stroma.[10]

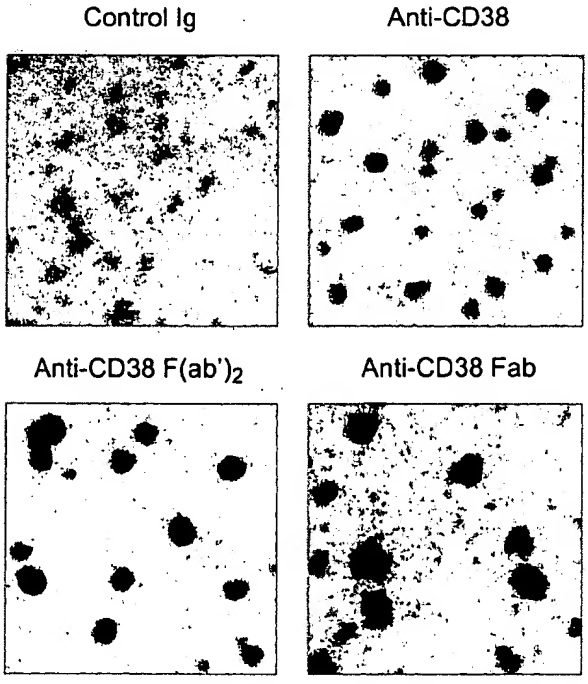

Figure 5. CD38 ligation induces aggregation of murine 32D cells tranfected with human CD38. Cultures of 32D expressing human CD38 were exposed for 24 hours to a nonreactive isotype-matched control Ig or to 3 preparations of anti-CD38 (THB7): whole Ig molecule, $F(ab')_2$ fragments, and Fab fragments. Cell aggregation was induced by anti-CD38 irrespective of the integrity of the Ig molecule. The loose aggregation seen with control Ig was also seen in cells cultured without antibody (not shown).

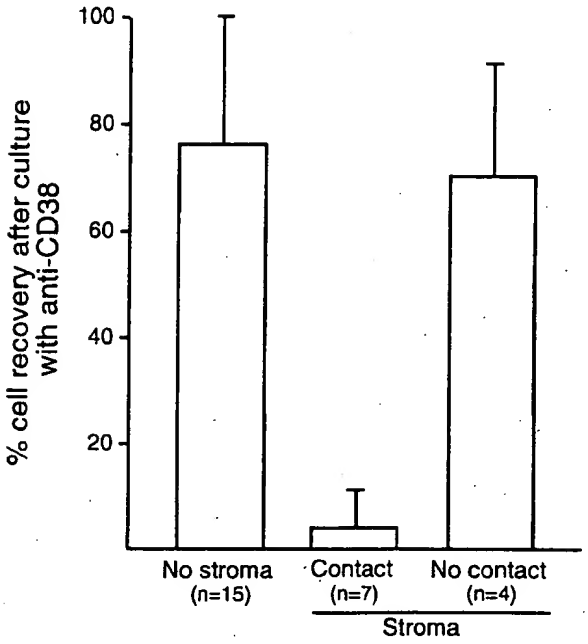

Figure 6. Direct contact with stroma enhances the suppressive effects of CD38 ligation in 32D cells expressing human CD38. Cells were cultured for 7 days without or with bone marrow-derived stromal layers. In the latter cultures, 32D cells were either in direct contact with stroma ("Contact") or separated from stroma by a 0.4-μm porous membrane ("No contact"). Results are expressed as mean (± SD) percent cell recovery in the presence of anti-CD38 (T16) as compared with parallel cultures with a control nonreactive Ig. The number of experiments for each culture condition is indicated.

The 32D cells do not require contact with stroma to remain viable. To determine whether direct contact with stroma was necessary for the enhanced response of the cells to anti-CD38, we prepared cultures in which 32D-CD38 cells were separated from stroma by a 0.4-μm microporous membrane, which allowed free flow of soluble factors but blocked direct cell–cell contact. In 4 experiments under these culture conditions, CD38 ligation produced a cell recovery of 68.3% ± 23.2%. This percentage of cell recovery was similar to that seen in parallel cultures in which 32D-CD38 cells were exposed to anti-CD38 in wells without stroma (see Figure 6). Thus, direct contact with stromal layers, rather than exposure to stroma-derived soluble factors, was critical to enhance the response of cells to CD38 ligation.

CD38-mediated signaling in 32D cells

To test whether CD38 ligation in transfected 32D cells could trigger signal transduction, we engaged CD38 with an anti-CD38 antibody (T16) and performed Western blots on cell lysates. The anti-phosphotyrosine antibody 4G10 detected tyrosine phosphorylation of several proteins after 1 minute of CD38 ligation. The effect was maximal 5 minutes after CD38 ligation and decreased progressively thereafter (Figure 7). The main tyrosine-phosphorylated proteins had molecular masses of approximately 68, 72, 110, and 140 kDa. By contrast, an isotype-matched nonreactive antibody and an antibody to murine CD44 (IM7), a molecule highly expressed on 32D cells (our unpublished observations), did not noticeably increase tyrosine phosphorylation. Another anti-CD38

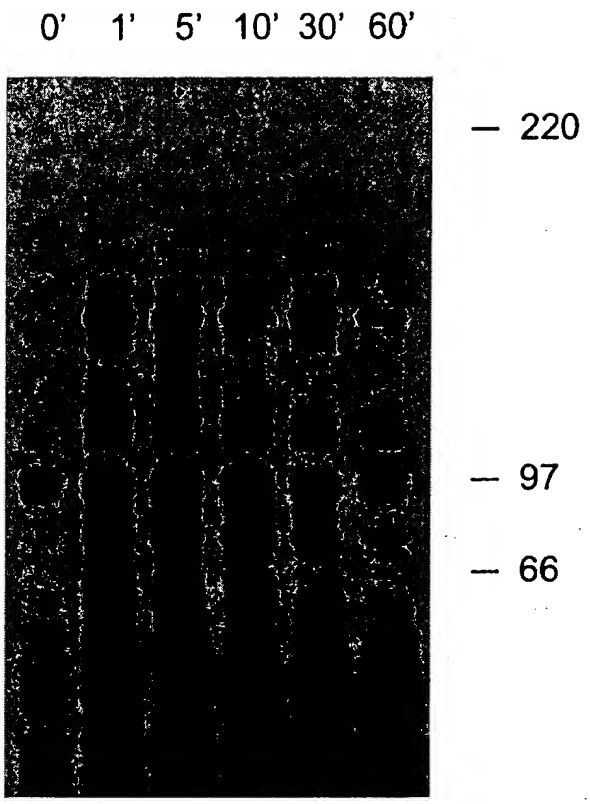

Figure 7. CD38 ligation in 32D cells expressing human CD38 induces tyrosine kinase activity. 32D cells were exposed to anti-CD38 (T16) for the times indicated. Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane, which was probed with the anti-phosphotyrosine antibody 4G10. Molecular mass markers (in kDa) are indicated.

antibody (THB7) did induce tyrosine phosphorylation, but its $F(ab')_2$ and Fab fragments did not.

We also tested whether CD38 ligation induced Ca^{++} flux in the same cells. A small but consistent increase in intracellular Ca^{++} was detected after CD38 ligation with T16, THB7, and THB7 $F(ab')_2$ and Fab fragments (Figure 8); this effect was not enhanced by culturing 32D cells on stroma before exposure to the antibody. Ca^{++} flux was abrogated by the addition of EGTA (5 mM) to the medium (see Figure 8). By contrast, an isotype-matched nonreactive antibody and the anti-CD44 (IM7) antibody did not alter the intracellular Ca^{++} content (see Figure 8).

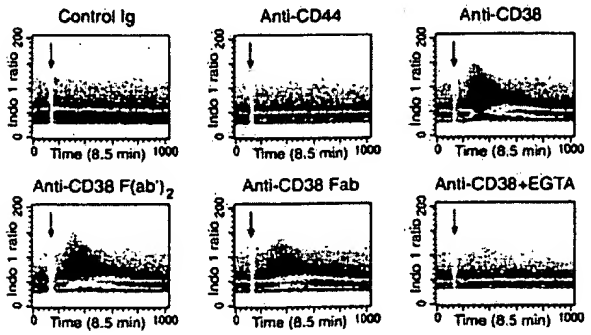

Figure 8. CD38 ligation in 32D cells expressing human CD38 induces Ca^{++} and influx. Cells were loaded with Indo-1. A shift in Indo-1 fluorescence was induced by the addition of anti-CD38 (THB7) as a whole Ig and $F(ab')_2$ and Fab fragments. The shift was abrogated by the presence of 5 mM EGTA and was not induced by an isotype-matched nonreactive or by an antibody to CD44, a surface molecule highly expressed in 32D cells.

Discussion

In this study, we found that CD38 ligation induces a marked inhibition of normal human myeloid cell differentiation. The suppressive effects of CD38 ligation on cell growth were particularly distinct among $CD34^-MPO^{++}$ cells (population 4), a phenotype that includes most promyelocytes and myelocytes.[28,31] Of note, both monocytic cells and granulocytic cells appeared to be affected by CD38 ligation. The human CD38 molecule could mediate suppressive signals even when ectopically expressed in murine myeloid cells, indicating that the signaling mechanism leading to this cellular effect is preserved across species. It is unclear whether the effect of CD38 was primarily caused by inhibition of cell proliferation or induction of apoptosis in normal immature myeloid cells. In cultures of leukemic myeloid cells derived from patients, however, induction of apoptosis was apparent.

Experiments by Inoue et al[17] with leukemic myeloid cell lines suggested that anti-CD38 antibodies could trigger signaling in myeloid cells by serving as a bridge between the CD38 molecule and FcγRII receptors. Although anti-CD38 antibodies lacking the Fc portion failed to induce measurable tyrosine kinase activity, all other cellular effects examined could be triggered irrespective of the integrity of the Ig molecule. One interpretation for this apparent discrepancy is that CD38-mediated tyrosine phosphorylation and cellular effects are independent. Although the former may require simultaneous engagement of CD38 and Fc receptors, the latter can be triggered by CD38 ligation alone. Alternatively, changes in affinity may have been introduced by the enzymatic removal of the Fc portion, leading to reagents incapable of causing a detectable surge in tyrosine kinase activity but still able to initiate all other cellular effects. Interestingly, anti-CD38 in a monomeric Fab fragment form also reduced cell recovery and caused aggregation, an effect we had previously observed in immature lymphoid cells.[27] These results are consistent with the model of CD38 extrapolated by Prasad et al[34] from the crystal structure of the *Aplysia californica* adenosine diphosphate ribose cyclase, a CD38 homolog. This model depicts CD38 as a dimer with a hinge motion that can effect signal transduction in response to external stimuli. We speculate that anti-CD38 antibodies can exert their cellular effects by eliciting this hinge motion, rather than by dimerization or oligomerization of the receptor.

Our observations appear to contradict those of Konopleva et al,[35] who found that CD38 ligation in myeloid cell lines and AML blast cells induced a variable increase in thymidine incorporation, colony formation, and cell numbers recovered after culture, and had no discernible effect on the numbers of normal granulocyte-monocyte colony-forming units. One critical difference between the studies is our addition of stromal feeder layers, which mimic the in vivo bone marrow microenvironment. Both normal hematopoietic cells and primary leukemic cells require stromal feeder layers for optimal in vitro growth.[36-42] In experiments with CD38-transfected 32D cells, we found that CD38 ligation had an inconsistent effect on cell recovery after culture without stroma but radically and consistently reduced cell numbers in the presence of stroma. The accessory effect of stroma-derived signal recalls our previous observations in human lymphoid leukemic cell lines.[10]

The reduced cell recovery observed in stroma-dependent cultures of normal and leukemic primary cells could conceivably have

been caused by interference with the adhesion of these cells to stromal elements. This possibility is suggested by the reported ability of CD38 to mediate adhesion of lymphocytes to endothelial cells,[43] and to bind to hyaluronic acid,[44] which is abundant in the extracellular matrix of stromal layer prepared in the presence of corticosteroids, such as those in our assay.[45] However, we think this possibility unlikely because CD38 ligation also suppressed cell growth in 32D and other cell lines[10] that do not require interaction with stroma for survival and proliferation. Thus, the mechanism by which CD38- and stroma-mediated signals interact and synergize remains to be clarified. It appears, however, that the relevant stroma-derived factors either are expressed on the surfaces of stromal cells or accumulate in the extracellular matrix, because separation of 32D cells and stroma by a microporous membrane markedly reduced the suppressive effects of CD38 ligation.

In summary, CD38 in conjunction with stromal elements directly mediates signals that culminate in suppression of myeloid cell development. The effects of CD38 ligation on myeloid cells described in this study have many analogies with the effects of the same stimulus in immature B lymphoid cells,[10,11,27] and suggest common signaling pathways between the 2 lineages. The physiologic role of CD38 at the early stages of hematopoietic cell differentiation remains an enigma, but its powerful signaling properties, suppression of cell growth, and heterogeneous distribution indicate that this molecule could be involved in the homeostasis of hematopoiesis within the bone marrow microenvironment. Although CD38-deficient mice have apparently normal hematopoiesis,[46] the distribution of CD38 differs in murine and human cells,[47,48] rendering comparisons between the 2 species difficult. Natural CD38 ligands, capable of inducing events similar to those triggered by anti-CD38 monoclonal antibodies, have not yet been identified. CD31 and hyaluronic acid can bind CD38,[43,44] but did not elicit any detectable biochemical or cellular response in vitro (unpublished observations).[27] Thus they cannot accout for the full spectrum of biologic effects mediated by CD38, suggesting the existence of other CD38 ligands. Nevertheless, our findings have important practical implications. The immunophenotype of human normal and leukemic stem cells is often defined as $CD34^+CD38^-$,[6,20,49-51] because the growth potential of sorted $CD34^+CD38^-$ cells is greater than that of $CD38^+$ cells. In light of the results of this and previous studies,[10] we propose that the lesser growth potential of sorted $CD38^+$ cells may be, at least in part, caused by signals mediated by anti-CD38 antibody used in the sorting process. We suggest that, in experiments designed to compare the growth of selected cell populations, cell sorting should be done using antibodies that have no effect on cell growth.

Acknowledgments

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Diagnostic Flow Cytometry

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This CME activity was planned and produced in

accordance with the ACCME Essentials.

This document is worth 1 CME credit hour. Total Study

Time = 1 hour.

Educational Objectives:

Upon completion of this activity, The participant should be able to:

- know the basic methodology behind diagnostic flow cytometry
- know how specimens are collected , processed and analyzed by flow cytometry
- understand how different patterns of immunophenotypes relate to specific leukemias and lymphomas
- understand the clinical uses of DNA cell cycle analysis and image analysis

Table of Contents

Test

Evaluation

Introduction

Flow cytometry is the quantitative multiparameter measurement of chemical or physical characteristics of cells in suspension. A flow cytometer measures as a cell passes through detectors 1) the cell's light scatter and 2) the electronic cell volume. It also measures as it passes through a fluorescent excitation beam the cell's 3) axial (at a right angle) light loss and 4)morphological information (derived from the cell shape or time duration of light scatter signals. By incubating cells with fluorescent-tagged antibodies the composition of antigens on the cell surface is determined (phenotype) and by incubating with a fluorescent dye which incorporates into the DNA the DNA composition is determined (ploidy).

In fluorescence, the exciting (incident) light is absorbed by the electron, which increases the energy of the electron and raisesit to a higher orbital. This electron is not stable at this higher energy level, however, and quickly reverts to the lower orbital in less than a second. During this reversion, the electron will give off some of its energy as light, which is thefluorescence. Since the amount of energy given up is not as much as input, the wavelength of the fluorescent light is longer (less energetic) than the incident light. The recent development of chromogens that are excitable by the standard argon laser has allowed bench top flow cytometers to routinely perform these analyses. In addition to allowing a more definitive typing of all leukocyte populations, multicolor analysis can decrease the number of tubes needed to run a panel of antibodies.

This new technology needs to be thoughtfully utilized in order to be both medically and economically efficient. This will serve as a concise review of flow cytometric techniques for established diagnostic and prognostic applications of antigen immunophenotyping of peripheral blood, bone marrows and tissues for leukemias and lymphomas. In addition, we will cover the prognostic usefulness of DNA cell cycle analysis in hematopoetic diseases and tumors.

Immunophenotyping

The process of measuring the types of antigens expressed on cell surfaces by flow cytometry is referred to as immunophenotyping. To detect these antigens, antigen- specific monoclonal antibodies are used which have been with a fluorescent dye or fluorochrome. The two most common fluorochromes used are phycoerythrin (PE) and fluorescein isothiocyanate (FITC). After incubating the cells with the specific antibodies of interest and washing away any unbound antibody, the cells are analyzed by flow cytometry which categorizes them by size, granularity, and fluorochrome inten sity as previously mentioned. A standardized nomenclature is used internationally to categorize the antibodies according to the antigen they detect. Each category is called a cluster of differentiation (CD) and is numbered. Before this standard was created, an antibody was named by each company or scientist who produced it. An **antibody list** is available of the CD numbers, the former antibody name, and the antibody s specificity.

Collection and Specimen Processing

Collection

The recommended method of collection is to collect blood by venipuncture into evacu ated tubes containing an appropriate anticoagulant. Use pediatric tubes for pediatric specimens and be sure the tube is filled until the vacuum is expended. Mix the blood well with the anticoagulant in the tube to prevent clotting. There are three different anticoagulants used in flow cytometry. If the specimens will be processed within thirty hours of collection then any of the three anticoagulants, potassium EDTA, acid citrate dextrose (ACD), or heparin, may be used. If the specimen will not be processed within thirty hours, then use either ACD or heparin.

Specimen Transport and Integrity

Maintain and transport all specimens for flow cytometry analysis at room temperature (18-22 degrees C). This is necessary to maintain the viability of the cells and their expression of antigens. Once the specimen arrives at the laboratory, inspect the specimen and take the following actions if necessary:

- If the specimen feels too hot or cold, but is not obviously frozen or hemolyzed, make a note on the worksheet. Light scatter abnormalities will be noted if the specimen has been harmed.
- Reject the specimen and request a recollection if the specimen is frozen, hemolyzed, over 48 hours old, or clotted.

Specimen Processing

A hematologic analysis for the white blood cell count and lymphocyte differential should be performed within six hours of collection. Generally this is done on an automated instrument, but if it "flaggs" the lymphocyte population, a manual differential should be performed.

For optimal results specimens should be processed immediately through fixation and the immunophenotype should be performed within 24 hours. The maximum time period for analysis is dependent on the collection agent used for the specimen and should be established by each individual laboratory. A general rule is that specimens can be processed for immunophenotype up to 30 hours after collection, but should not be analyzed after 48 hours. A direct two-color immunoflourescent whole blood lysis method is used (better known as the "stain, then lyse" procedure). **Video 1.**

Tubes are incubated in the dark to maximize fluorescence capability. **Video 2.** When centrifuging the specimen during the wash step, maintain the centrifugation forces between 300 and 400 g for 3 to 5 minutes. Vortex the sample to mix up the antibodies with the cells and break up cell aggregates (Immediately before analysis, vortex the specimen again to optimally disperse the cells). A source of protein is included in the wash buffer to reduce cell clumps and autofluorescence Before analysis, fix the sample with either 1-2% buffered paraformaldehyde or formaldehyde. **Video 3.** The fixatives should always be made from electron-microscopy grade aqueous stock and be made fresh weekly. Store all stained samples in the dark and at refrigerator temperatures (4- 10 degrees C) until analysis.

Positive Controls For Immunophenotyping

A positive control is used to determine whether specimen processing is optimal. Generally a whole blood specimen is used which is obtained from a donor who ideally matches the patient population analyzed in the laboratory. If the control falls outside of the established normal ranges for the laboratory then the reason must be determined. Examples of reasons for positive control failure might include poor lysis or poor labeling.


The use of a HLE/Leu M1 control helps to spot the monocytes for gating. In addition to the daily positive controls, there should always be a positive control used to check new lots of reagents or when the labeling efficiency of the current lot is questioned. New reagents must demonstrate similar results to previous reagents of known acceptable performance.

Peripheral Blood Analysis For CD4/CD8

Generally the highest volume test in a flow cytometry laboratory is a CD4/CD8 analysis of the peripheral blood performed in patients suffering from human immunodeficiency virus (HIV) infection. In acquired immunodeficiency syndrome the HIV-1 infection causes a decrease in the T-lymphocyte population containing the CD4 receptor. The percentage of CD4 positive (CD4+) T-cells circulating in the peripheral blood has been shown to have a direct correlation to the severity of illness in the patient. The lower the percentage of CD4+ cells then the more likely the patient will develop a serious infec tion. For this reason, prognostic indicators and clinical decision points have been established on certain levels of CD4+ T-cells. Therefore, a CD4/CD8 analysis is performed approximately every 3-6 months to closely follow these patients.

The CDC has recommended the following panel to be performed for peripheral blood lymphocyte analysis of CD4/CD8.

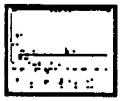
Recommended CD4/CD8 Panel

FITC	PE	REASON FOR USING
CD45	CD14	For gating, lymphocytes are brightly positive for CD45 and negative for CD14
Isotype	Isotype	Control to set cursors, or discriminators, for positivity in the samples to follow
CD3	CD4	To measure CD4+ T cells; only CD3+/CD4+ cells

		should be reported as CD4+ cells
CD3	CD8	To measure CD8+ cells; only CD3+/CD8+ cells should be reported as CD8+ cells. CD3-/CD8+ cells are natural killer cells
CD3	CD19	To measure B cells to account for all lymphocytes and for quality assurance
CD3	CD16 CD56	To measure natural killer cells CD16+/CD56+/CD3- for quality assurance, to account for all lymphocytes

The isotype control serves to detect nonspecific binding of the mouse monoclonal antibody to cells. An antibody with no human specificity is always used, but the antibody should always be of the same isotype as the panel antibodies. Also it is used for setting markers to separate fluorescent negative and positive populations. Besides helping to determine the T cell populations, CD3 serves as an internal control which will demonstrate any tube to tube variability. The CD3 positive results should have only a 3% variability. If the value is greater then 3%, then the antibody combination should be restained and analyzed.

The use of the CD3/CD19 is to determine the number of B cells.



The measurement of CD4+ cells is reported as a percentage and an absolute number. To calculate the absolute CD4+ T-cells it is necessary to perform a white blood cell (WBC) count and a differential which determines the percentage of WBCs that are lymphocytes. The following calculations can then be made and reported:

Absolute lymphs = WBC count x % lymphs

Absolute CDx = Absolute lymphs x % CDx

(where x = a number)

Lymphocyte Subpopulations Identified By Antibody Combinations

COMBINATION	POPULATION IDENTIFIED
CD4+/CDw29+	Helper/effector, more mature memory cells
CD4+/CD45R+	Suppressor inducer, less mature nonmemory cells
CD4+/Leu8+	Suppressor inducer, some helper function

CD4+/Class II MHC	Activated cells, immature cells
CD4+/CD25+	Activated cells (IL2 receptor)
CD4+CD38+	Immature cells, activated cells
CD8+/CD11b+	Of the CD11b+ cells the suppressors are bright CD8+ and NK are dim CD8+
CD8+/CD28+	Cytotoxic precursor/effector cells
CD8+/CD57+	Cytotoxic function
CD8+/Class II MHC+	Activated cells, immature cells
CD8+/CD25+	Activated cells (IL2 receptor)
CD8+/CD38+	Immature cells, activated cells
CD16+/CD57+	Low NK activity
CD16+/CD56+	Most potent NK activity

Leukemia Immunophenotyping

Leukemias are clonal proliferations of the bone marrow hematopoietic cells arrested at a discrete stage of maturation which replace the normal marrow cells. The degree of maturity achieved by these cells and the cell lineage involved are used to classify leukemias into acute (less mature) or chronic (more mature). Either lymphocytic or myelocytic hematopoietic cell lines are usually involved. Acute leukemias are rarely seen of the megakaryocytic or erythrocytic cell lines.

For acute leukemias, immunophenotyping can be supportive evidence in determining the lineage of the leukemic cell. It is important to note that there is no leukemic specific marker and it is the composite phenotype that must be interpreted. Commonly used markers for leukemias are available.

The acute leukemias are broken into two major groups, acute lymphoblastic leukemia and acute non-lymphocytic leukemia (contains the myeloid leukemias, erthryocytic leukemias, and megakaryocytic leukemias).

Acute Lymphoblastic Leukemia (ALL)

In children under 15 years of age, acute lymphoblastic leukemia (ALL) is the most common malignancy. The incidence of ALL peaks between 3 to 5 years of age, but can be seen in infants under one year of age and in young adults (comprises approximately 20% of adult leukemia cases). The cure rate is more favorable in children between 1 and 10 years of age than in adults. Over half of children with favorable prognostic factors are cured and 85% achieve remission. The morphologic classification is based on the light microscopic appearance which is distinctive with nuclear pleomorphism, 1-2 indistinct nucleoli, usually a high nuclear to cytoplasmic ratio, and coarse nuclear chromatin. The cytochemical stain, myeloperoxidase, is absent.

Most ALL cases are B-cell proliferations (approximately 75-85%) but can very in their degree of maturity. Usually the leukemias arise from very early B-cells. Initial diagnosis relies upon two monoclonal antibodies, CD19 and HLA-DR. The CD19 antigen is the earliest B-cell specific antigen and precedes the expression of HLA-DR (which is not specific to the B-cell lineage). Other monoclonal antibodies can be useful in classifying B-cell leukemia, CD10, CD20, CD21, and CD24,

cytoplasmic immunoglobulins, and surface immunoglobulins. CD21, a mature B-cell marker, is generally not expressed in B-cell ALL nor are T-cell antigens. Remember if surface markers of more than one cell line is present the possibility of a mixed or biphenotypic leukemia should be considered.

The most common B-cell ALL phenotype (approximately 50% of cases) expresses CD10 (common acute lymphoblastic leukemia antigen, cALLa), CD19, CD20, terminal deoxynucleotidyl transferase (TdT), and HLA-DR and does not express cytoplasmic and surface immunoglobulin. This subtype of B-cell ALL has the most favorable prognosis. The next most common (approximately 30% of cases) expresses all of the above with the exception of CD20. Rarely, ALL cases express just CD19 alone or CD19, CD20, HLA-DR, CD10, and either surface or cytoplasmic immunoglobulin. TdT can be present in either B or T-cell ALL. HLA-DR can be helpful to differentiate immature from mature B-cells since it has a more intense reaction in immature B-cells.

A leukemic manifestation of Burkitt s lymphoma which is a very aggressive disease is a morphologically distinct form of B-cell ALL. It is characterized by large uniform cells, prominent nucleolus, and moderately abundant deep basophilic cytoplasm which may have small vacuoles. These vacuoles stain Oil Red O positive. These cells have a B- cell immunophenotype with detectable surface immunoglobulin. Burkitt s has the worst prognosis of ALL.

T-cell ALL is seen in approximately 15-25% of cases and tends to be a more aggressive disease. The typical clinical presentation is an older male who has a high blast count in the peripheral blood and mediastinal masses. The diagnosis of T-cell ALL by phenotyping is much more difficult than B-cell ALL for several reasons.

- In T-cell ALL there is no marker for monoclonality such as the immunoglobulin light chain (kappa or lambda) in B-cell ALL.
- There are few markers which are detected only in the early phases of T-cell development and these markers occur uncommonly in T-cell ALL.
- HLA-DR is usually absent in T-cell ALL.
- Benign proliferations can be seen and T-cells are normally prominent in most specimen types (60-80% of the lymphocytes).

The most sensitive antibody is CD7 which is directed to the pan-T antigen. Approximately 95% of T-cell ALL will express CD7 and a diagnosis of T-cell should be examined closely if CD7 is not expressed. However CD7 alone is not a marker specific for T-cell ALL since it has been reported to be aberrantly expressed in B-cell ALL and acute myeloid leukemias. Other useful markers in T-cell ALL are CD1a (except in thymus where thymocytes normally express CD1a), CD2, CD3, and CD5. CD5 and CD2 are expressed in most cases of T-cell ALL.

Acute Non-Lymphocytic Leukemia

Acute non-lymphcytic leukemia (ANLL) is composed of acute myeloid leukemia (AML) and the rarer leukemias acute erythrocytic and acute megakaryocytic leukemia. Immunophenotyping is the most useful in differentiating AML from ALL and has not been particularly useful in subclassifying AML or for prognostic purposes. AML is most commonly seen in adults between the ages of 15 and 40 years of age. Only about 20% of childhood leukemias are AML.

Light scattering properties can be very useful in identifying the blast cells of AML which tend to be found on forward versus orthogonal scatter as well circumscribed population about the location where normal lymphocytes are seen. Since bone marrow is very heterogeneous in nature, the finding of a single-cell population of similar size and characteristics should be very suspicious for a malignancy. An antibody is considered positive if greater than 20% of the cells express the antigen.

Immunophenotypic classification of AML does not very well match the strict morphologic classifications currently used by pathologists. A panel of specific antibodies including CD13, CD14, CD33, and CD34 is used to indicate if the cells are of a myeloid lineage and then whether there is any degree of monocytic lineage involvement. Also antibodies such as HLA-DR and CD45 are included. These antibodies are not myeloid lineage specific, but are generally seen in AML. The lack of expression of HLA-DR found in most acute progranulocytic leukemias (APL), a subtype of AML, is useful in its diagnosis.

In all forms of AML, the demonstration of the lack of of expression of lymphocyte specific markers is helpful by means of exclusion. However, the presence of these markers does not necessarily exclude the possibility of AML or a biphenotypic leukemia. CD7 is found in up to 25% of AML cases.

CD14 is virtually restricted in its expression to mature monocytes. Therefore it is useful to separate monocytes from blasts or monocyte precursors. CD11c along with CD14 expression can be very useful in detecting acute myelmonocytic and acute monocytic leukemias since it is generally not expressed in other forms of AML or ALL.

For the rarer cases of acute erythrocytic leukemia the use of anyiglycophorin can be helpful in diagnosis. In acute megakaryocytic leukemia anti-platelet glycoprotein antibodies allows for their identification.

Chronic Leukemias

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a disease of older adults, 50-70 years of age, and occurs more commonly in males than females (a 2:1 ratio). CLL is commonly discovered on a routine peripheral blood smear. The disease is a clonal expansion of an immunologically competent lymphocytes involving the bone marrow, lymph nodes, and or spleen. The disease is almost indistinguishable from well differentiated lymphoma and is generally referred to as CLL if the bone marrow is extensively involved. If the disease is primarily of the lymph node or spleen then the term well differentaited lymphoma or small lymphocytic lymphoma is used. Morphologically the cells are indistinguishable from normal mature lymphocytes.

The majority of CLL are monoclonal B-cells which have a monotypic expression of either kappa or lambda light chains and react with CD5. The staining intensity of CLL with CD5 is usually greater than either with kappa or lambda light chains. This finding can be helpful in distinguishing CLL from a reactive lymphocytosis.

Prolymphocytic Leukemia

Prolymphocytic leukemia is a more aggressive form of CLL. Like CLL pan-B cell markers are positive (CD19, CD20,

CD22, and CD24). The major distinguishing finding is that the monotypic surface immunoglobulin staining is present in high density.

Hairy Cell Leukemia

Hairy cell leukemia (HCL) is a rare leukemia composed of B-cells with a slightly less mature morphology and a distinctive appearance with cytoplasmic projections. HCL reacts with pan-B cell markers (CD19, CD20, CD22, and CD24) but when compared to CLL it has increased surface immunoglobulin staining.

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) occurs primarily in middle aged adults although can be seen to a lesser extent in other age groups. CML is a disease of a multipotential stem cell and thus involves more than one lineage. Immunophenotyping is not generally useful in CML, except when the disease evolves into a more aggressive form known as blast crisis. In blast crisis immunophenotyping is performed to determine the lineage of the blast cell proliferation involved. Interpretation is similar to ANLL and ALL.

Lymphoma Immunophenotyping

Another common test for the flow cytometry laboratory is immunophenotyping to rule out lymphomas. The essential difference between reactive and malignant lymphoid proliferations is the expansion of a clonal population in the latter. Generally, non-Hodgkin's lymphomas are monoclonal (derived from a single cell). If it is derived from a B-cell all of the progeny will exhibit restricted light chain expression (kappa or lambda) or no light chain expression (25%).

If the malignancy is derived from a T-cell, it is more difficult to establish the presence of maligancy due to the lack of phenotypic markers of T-cell clonality. Therefore, immunophenotypic evidence of T-cell malignancies rests on abnormal/aberrant antigen expression and or the presence of a DNA abnormality. In summary, to arrive at a diagnosis of lymphoid neoplasm by flow cytometry the case must show 1) immunophenotypic abnormalities and/or 2) abnormal DNA ploidy (reflective of an increase or decrease in chromosome number).

Immunophenotyping in Hodgkin s lymphomas are more useful in ruling out a diagnosis of Hodgkin s disease than in establishing a diagnosis. In general, it is almost impossible to distinguish a Hodgkin s lymphoma from a benign T-cell proliferation. Small lymphocytic lymphomas are maily (98%) of B-cell origin and express the B cell associated markers (CD19, CD22, and CD24). The rare T-cell small lymphocytic lymphomas usually express a mature T-cell helper immunophenotype (expressing CD2, CD3, CD4, CD5, CD7, and not expressing CD8). Follicular lymphomas are of B cell origin and express surface immunoglobulin,most of the pan-B cell markers, and very frequent CD10 may be positive.

Quality Assurance of Immunophenotyping
DNA Cell Cycle Analysis

SECOND EDITION

CELLULAR AND MOLECULAR IMMUNOLOGY

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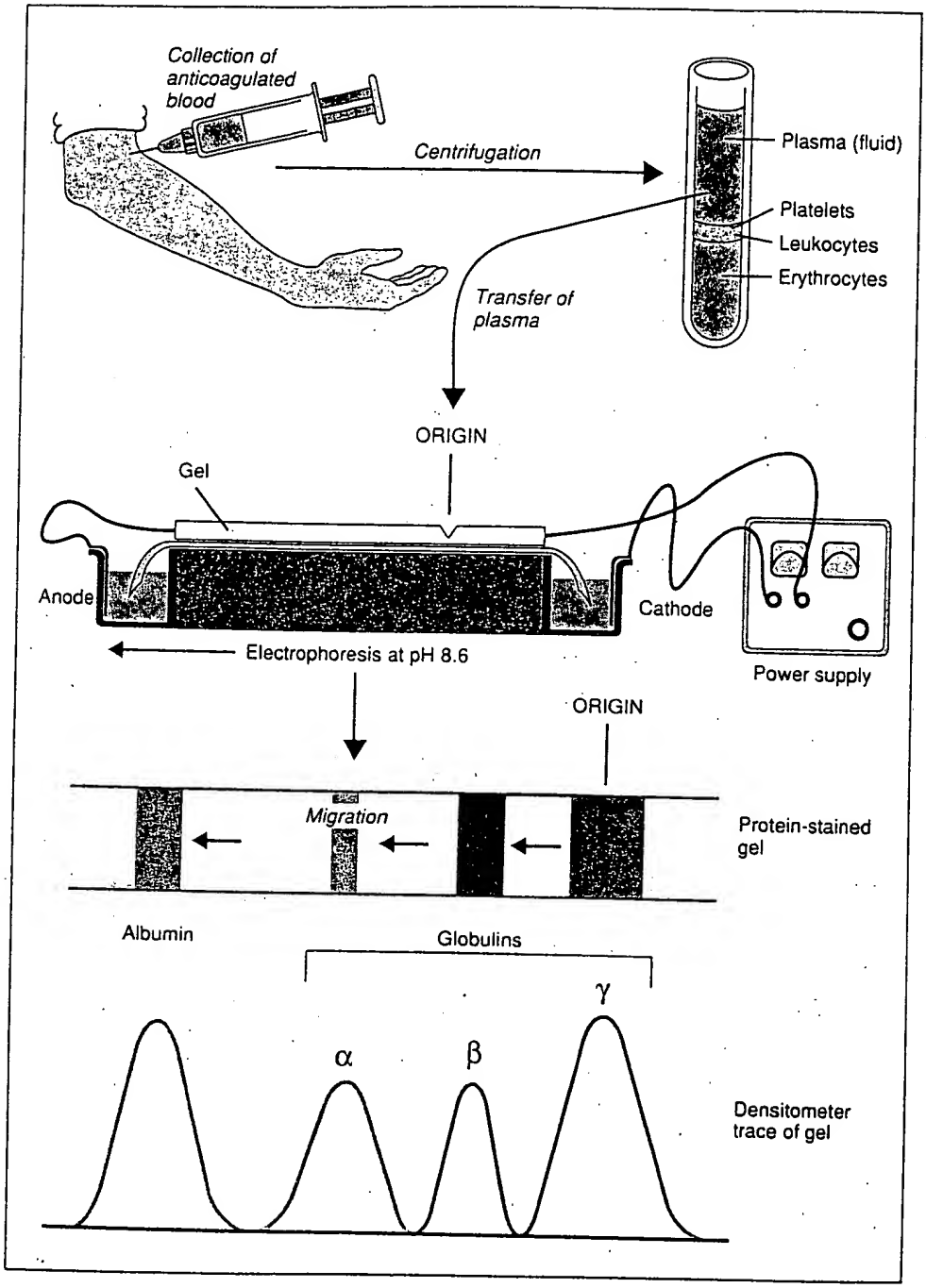

FIGURE 3–1. Separation of plasma proteins by electrophoresis. *Electrophoresis separates plasma proteins into albumin and globulins. Most antibodies are found in the γ globulin fraction.*

rapid and highly resolved separations. This technique is called *high-pressure liquid chromatography* (HPLC).

Overview of Antibody Structure

A number of the structural and functional features of antibodies were determined from the early studies of these molecules:

1. *All antibody molecules are similar in overall structure, accounting for certain common physicochemical features, such as charge and solubility.* These common properties may be exploited as a basis for the purification of antibody molecules from fluids such as blood. *All antibodies have a common core structure of two identical light chains (each about 24 kilodaltons [kD]) and two identical heavy chains (about 55 or 70 kD)* (Fig. 3–2). One light chain is attached to each heavy chain, and the two heavy chains are attached to each other. Both the light chains and the heavy chains contain a series of repeating, homologous units, each about 110 amino acid residues in length, which fold independently in a common globular motif, called an **immunoglobulin domain.** All Ig domains contain two layers of β-pleated sheet with three or four strands of antiparallel polypeptide chain. Certain Ig domains, such as those comprising variable regions (see later), have

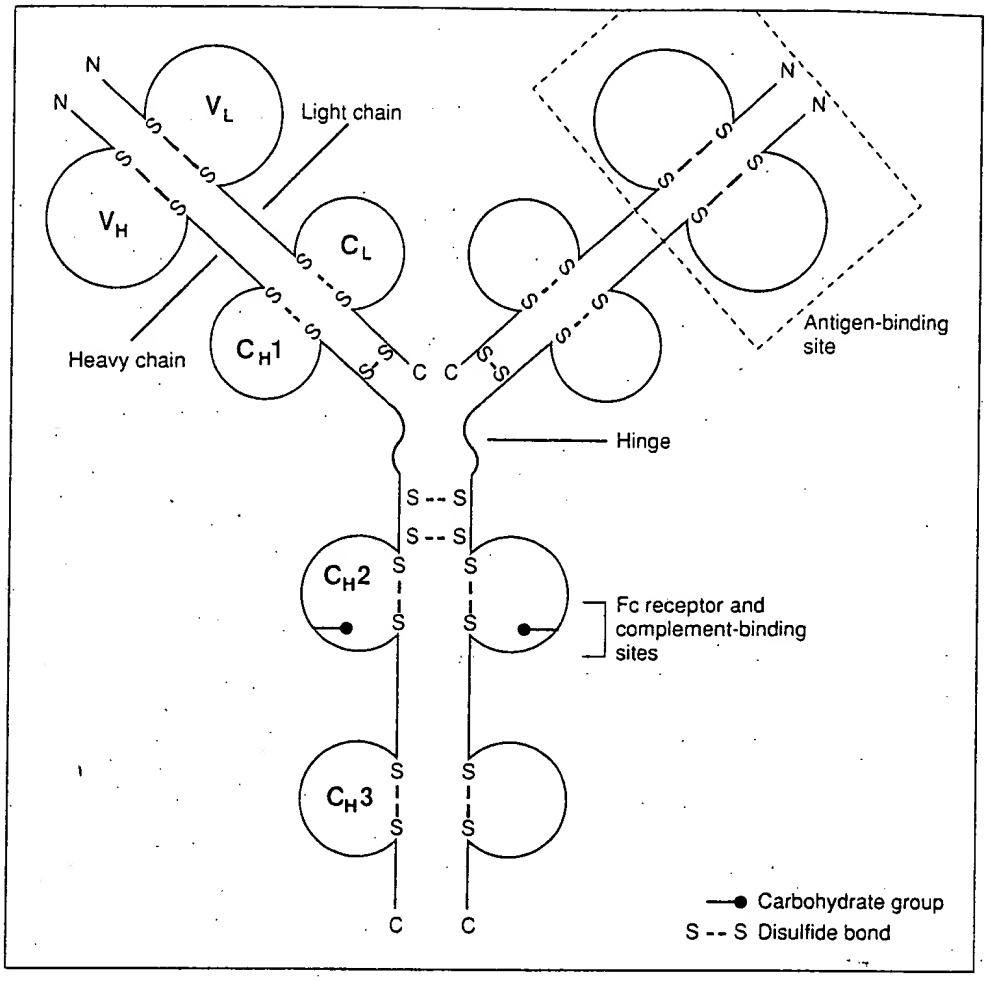

FIGURE 3–2. Schematic diagram of an immunoglobulin molecule. *In this drawing of an IgG molecule, the antigen-binding sites are formed by the juxtaposition of V_L and V_H domains. The locations of complement and Fc receptor–binding sites within the heavy chain constant regions are approximations. S--S refers to intrachain and interchain disulfide bonds; N and C refer to amino and carboxy termini of the polypeptide chains, respectively.*

an extra strand in each of the two layers. As will be discussed in Chapter 7, many other proteins of importance in the immune system contain regions that use the same folding motif and show structural relatedness to Ig amino acid sequences. All molecules that contain this motif are said to belong to the **Ig superfamily,** and all of the gene segments encoding the Ig-like domains are believed to have evolved from the same common ancestral gene (see Chapter 7, Box 7–2).

2. Despite their overall similarity, *antibody molecules can be readily divided into a small number of distinct classes and subclasses, based on minor differences in physicochemical characteristics such as size, charge, and solubility and on their behavior as antigens* (Box 3–2). In humans, the classes of antibody molecules are called IgA, IgD, IgE, IgG, and IgM, and members of each class are said to have the same **isotype** (Table 3–1). IgA and IgG isotypes can be further subdivided into closely related subclasses, or subtypes, called IgA1 and IgA2, and IgG1, IgG2, IgG3, and IgG4, respectively. In certain instances, it will be convenient to refer to studies of mouse antibody. Mice have the same general isotypes as humans, but the IgG isotype is divided into the IgG1, IgG2a, IgG2b, and IgG3 subclasses in mice. The heavy chains of all antibody molecules of an isotype or subtype share extensive regions of amino acid sequence identity but differ from antibodies belonging to other isotypes or subtypes. Heavy chains are designated by the letter of the Greek alphabet corresponding to the overall isotype of the antibody: IgA1 contains $\alpha 1$ heavy chains; IgA2, $\alpha 2$; IgD, δ; IgE, ϵ; IgG1, $\gamma 1$; IgG2, $\gamma 2$; IgG3, $\gamma 3$, IgG4, $\gamma 4$; and IgM, μ. The shared regions of heavy chain amino acid sequences are responsible for both the common physicochemical properties and the common antigenic properties of antibodies of the same isotype. In addition, the shared regions of the heavy chains provide members of each isotype with common abilities to bind to certain cell surface receptors or to other macromolecules like complement and thereby activate particular immune effector functions. Thus, the separation of antibody molecules into isotypes and subtypes on the basis of common structural features also separates antibodies according to which set of effector functions they commonly activate. In other words, *different effector functions of antibodies are mediated by distinct isotypes and subtypes.* As we shall see later, there are two isotypes of antibody light chains, called κ and λ. The light chains, however, do not mediate or influence the effector functions of antibodies.

3. *There are more than 1×10^7, and perhaps as many as 10^9, structurally different antibody molecules in every individual, each with unique amino acid sequences in their antigen-combining sites.* This extraor-

BOX 3–2. ANTI-IMMUNOGLOBULIN ANTIBODIES

Antibody molecules are proteins, and therefore can be antigenic. Immunologists have exploited this fact to produce antibodies specific for Ig molecules that can be used as reagents to analyze the structure and function of Ig molecules. In order to obtain an anti-antibody response, it is necessary that the Ig molecules used to immunize an animal be recognized, in whole or in part, as foreign. The simplest approach is to immunize one species (e.g., rabbit) with Ig molecules of a second species (e.g., mouse). Populations of antibodies generated by such cross-species immunizations are largely specific for epitopes present in the constant regions of light or heavy chains. Such sera can be used to define the isotype of an antibody.

When an animal is immunized with Ig molecules derived from another animal of the same species, the immune response is confined to epitopes of the immunizing Ig that are absent or uncommon on the Ig molecules of the responder animal. Two types of determinants have been defined by this approach. First, determinants may be formed by minor structural differences (polymorphisms) in amino acid sequences located in the conserved portions of Ig molecules. Ig genes that encode such polymorphic structures are inherited as mendelian alleles. (The concepts of polymorphism and allelic genes are discussed more fully in Chapter 5.) Determinants on Ig molecules that differ among animals that have inherited different alleles are called allotopes. All antibody molecules that share a particular allotope are said to belong to the same allotype. Most allotopes are located in the constant regions of light or heavy chains, but some are found in the framework portions of variable regions. Allotypic differences have no functional significance, but they have been important in the study of Ig genetics. For example, allotypes detected by anti-Ig antibodies were initially used to locate the position of Ig genes by linkage analysis. In addition, the remarkable observation that in homozygous animals, all of the heavy chains of a particular isotype (e.g., IgM) share the same allotype even though the V regions of these antibodies have different amino acid sequences provided the first evidence that the constant portions of all Ig molecules of a particular isotype are encoded by a single gene that is separate from the genes encoding V regions. As will be discussed in Chapter 4, we now know that this surprising conclusion is correct.

The second type of determinant on antibody molecules that can be recognized as foreign by other animals of the same species is that formed largely or entirely by the hypervariable regions of an Ig variable domain. When a homogeneous population of antibody molecules, (e.g., a myeloma protein or a monoclonal antibody) is used as an immunogen, antibodies are produced that react with the hypervariable loops. These determinants are recognized as foreign because they are usually present in very small quantities in any given animal (i.e., at too low a level to induce self-tolerance (see Chapter 19)). Such determinants on individual antibody molecules are idiotopes, and all antibody molecules that share an idiotope are said to belong to the same idiotype. The term idiotype is also used to describe the shared idiotope. As will be discussed in Chapter 4, hypervariable sequences that form idiotopes arise both from inherited germline diversity and from somatic events. Individual idiotopes that arise from somatic events are rare and may define the products of one or a few clones of antibody-producing B cells. Idiotopes that arise from the germline are less rare and in some cases may be present on the majority of antibody molecules that recognize a particular antigen (dominant idiotopes). Unlike allotopes, idiotopes may be functionally significant because they may be involved in regulation of B cell functions. The theory of lymphocyte regulation through antibody-binding idiotopes expressed on membrane Ig molecules, called the network hypothesis, is discussed further in Chapter 10.

In addition to experimentally elicited anti-Ig antibodies, immunologists have also been interested in naturally occurring anti-bodies reactive with self Ig molecules. Small quantities of anti-idiotypic antibodies may be found in normal individuals. Anti-Ig antibodies are particularly prevalent in an autoimmune disease called rheumatoid arthritis (see Chapter 20), in which setting they are known as rheumatoid factor. Rheumatoid factor is usually an IgM antibody that reacts with the constant regions of self IgG. The significance of rheumatoid factor in the pathogenesis of rheumatoid arthritis is unknown.

dinary diversity of structure (whose generation is explained in Chapter 4) accounts for the extraordinary specificity of antibodies for antigens, because each amino acid difference may produce a difference in antigen binding. In theory, such extensive sequence diversity poses a structural problem because the three-dimensional structure of any protein is completely determined by its amino acid sequence and certain sequences are incapable of folding into soluble, stable proteins. In an antibody molecule, this problem is

TABLE 3–1. Human Antibody Isotypes*

Antibody	Subtypes	H Chain (Designation)	H Chain Domains (Number)	Hinge	Tail Piece	Serum Concentration (mg/ml)	Secretory Form	Molecular Weight of Secretory Form (kD)
IgA	IgA1	$\alpha 1$	4	Yes	Yes	3	Monomer, dimer, trimer	150, 300, or 400
	IgA2	$\alpha 2$	4	Yes	Yes	0.5	Monomer, dimer, trimer	150, 300, or 400
IgD	None	δ	4	Yes	Yes	Trace	—	180
IgE	None	ϵ	5	No	No	Trace	Monomer	190
IgG	IgG1	$\gamma 1$	4	Yes	No	9	Monomer	150
	IgG2	$\gamma 2$	4	Yes	No	3	Monomer	150
	IgG3	$\gamma 3$	4	Yes	No	1	Monomer	150
	IgG4	$\gamma 4$	4	Yes	No	0.5	Monomer	150
IgM	None	μ	5	No	Yes	1.5	Pentamer	950

* Multimeric forms of IgA and IgM are associated with J chain via the tail piece region of the heavy chain. IgA in mucus is also associated with secretory piece.

METHODS IN MOLECULAR BIOLOGY™

Flow Cytometry Protocols

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Preface

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2

Detection of Terminal Transferase in Leukemia

Elisabeth Paietta

1. Introduction

The mere presence of terminal deoxynucleotidyl transferase (TdT), a DNA polymerase, in leukemic cells provides no help in assigning these blast cells to a particular cell lineage *(1)*. Differential levels of TdT gene transcription, however, result in diagnostically significant expression patterns of the enzyme with lower biochemical activity and weaker staining intensity by antibody recognition in myeloid as compared to lymphoid leukemia *(2–4)*. One major advantage of measuring TdT by flow cytometry lies in its ability to objectively reflect staining intensities, a challenging task otherwise when one evaluates antibody staining under the microscope using the standard slide technique, thereby alleviating the need for cumbersome and expensive biochemical enzyme assays. The weak fluorescence staining of TdT-expressing myeloid leukemia cells, however, until recently has caused significant technical problems in the flow cytometric TdT detection, whereas several approaches have proven successful in the flow cytometric evaluation of TdT in the intensely staining lymphoid cells *(3)*. Using optimal experimental conditions, the combined analysis of nuclear TdT and surface antigens in all types of leukemia now allows for the detection of minimal residual disease at levels as low as 0.02–0.5% of abnormal cells.

Although in normal hematopoiesis TdT is detected predominantly in cortical thymocytes, with few (<5%) bone marrow cells (originally termed "prothymocytes"), and none of peripheral blood cells expressing appreciable TdT activity *(5)*, TdT has been convincingly demonstrated in lineage–antigen-negative, $CD34^+$-normal bone marrow progenitor cells *(6)*, identifying this enzyme as a lineage-uncommitted hematopoietic marker. The occurrence of TdT in lymphoid malignancies is uncontested, with highest levels of the

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3. 0.1% Triton X-100: weigh 0.1 g of Triton X-100 (use a dropper for this viscous solution) into 100 mL distilled water; stir until the Triton is dissolved.
4. Aldehyde blocking buffer: 3.75 g glycine, 10 g sucrose, in 500 mL of 1X PBS.
5. FACS-lysing solution: the 10X solution is commercially available from Beckton-Dickinson; dilute 1:10 in distilled water before use.
6. PBS/BSA/azide: dissolve 2–5 g (according to your own preference) of bovine serum albumine (BSA) and 0.1 g of sodium azide in 100 mL of 1X PBS.
7. Immunofluorescence assay medium (IFA) (*see* **Note 3**): 10 m*M* HEPES, pH 7.4, 150 m*M* NaCl, 4% calf serum (heat inactivated at 65°C for 30 min). Prepare 1 *M* HEPES solution, pH 7.4 (260.3 g/L of distilled water) and a 1.5 *M* NaCl solution (87.7 g/L of distilled water). For 100 mL of IFA, mix 1 mL of 1 *M* HEPES, pH 7.4, 10 mL of 1.5 *M* NaCl and 4 mL calf serum, and add 85 mL of distilled water.
8. Fix & Perm: the solutions are commercially available from Caltag.

2.2. Antibody Sources

Either a mixture of FITC-conjugated mouse monoclonal anti-human TdT immunoglobulins or a single FITC-conjugated monoclonal anti-TdT antibody is recommended. From the information available, good experiences have been reported with the antibodies distributed by Supertechs, (Bethesda, MD); Dako, (Carpinteria, CA), or Immunotech. It is important to use FITC-conjugated mouse monoclonal immunoglobulins with irrelevant specificity as negative controls. If unconjugated anti-TdT antibody is used, counterstaining with FITC-conjugated secondary immunoglobulin is performed following standard procedures. It is recommended to test for antibody specificity and suitability in your own test system using known TdT-positive and -negative control cells (*see* **Note 4**).

3. Methods

This section summarizes the various protocols described for flow cytometric TdT staining and focuses on discussing their technical and diagnostically relevant advantages and disadvantages. Methodological details for proven satisfactory procedures in both myeloid and lymphoid leukemia are presented.

3.1. The FACS-Lysing Solution (BD) Procedure (17,19)

Although not proven to be reliable in TdT staining of myeloid leukemia cells, this method is discussed because of its cost effectiveness and because it is a procedure routinely used for red cell lysis prior to acquisition of samples on the flow cytometer. It can be applied for whole blood or bone marrow as well as for mononuclear cells isolated by ficoll density gradient centrifugation.

1. Adjusted cell concentration to between 5.0 and 10.0 × 10^6/mL of IFA.
2. Combined staining for surface antigens will be discussed in the next section.

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Detection of Intracellular/Intranuclear Antigens

Applications in Leukemia/Lymphoma Analysis

Raymond H. Widen

1. Introduction

The increased utilization of flow cytometry for the study of cell-associated antigens has paralleled the continuing development of new monoclonal antibodies (MAbs) specific for cell-associated antigens. Availability of these reagents, combined with the introduction of newer fluorochromes such as phycoerythrin (PE) and peridinin chlorophyll (PERCp) or tandem conjugates such as PE-Cy5 that may be used in conjunction with fluorescein isothiocyanate (FITC) allow simultaneous measurement of coexpression of three or four different antigens. Flow cytometry instrumentation also has evolved to the point that three- and four-color analysis is routinely available in simple benchtop instruments, not just on high powered sorting instruments.

Although clinical flow cytometry often is associated simply with determining CD4 counts in HIV-infected individuals, multicolor flow cytometry has become an important tool for phenotyping leukemia or lymphoma samples in many laboratories. Indeed, the broad array of markers available to identify cell types and subtypes allows for precise identification of specific lineages, along with providing a means to detect abberant expression of lineage markers on cells that would not normally express such antigens (e.g., CD19 on acute myelogenous leukemia cells). Generally speaking, the vast majority of markers applied to leukemia/lymphoma typing to date have been cell surface proteins. Indeed, although specific markers for a variety of intracellular antigens are available from a variety of commercial sources, they have been underutilized because of some of the difficulties encountered in using flow cytometry to

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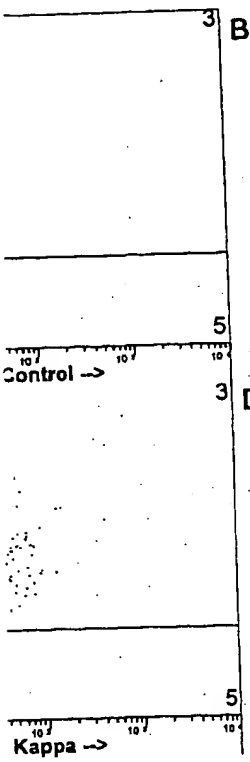

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cies as the primary antibody is added (for approx 10–15 min) to block any free binding sites on the labeled detecting antibody. After an additional wash step, staining with the second or third color reagents may begin with the target being either intracellular or on the cell's surface. The staining process now proceeds as described in **Subheading 3.** An alternative approach that has been described for analysis of two markers using two unlabeled primary antibodies involves the use of secondary antibodies specific for subclasses of the primary antigen-specific immunoglobulins. For example, if the primary monoclonal antibodies are of IgG1 and IgG2a types, one may perform follow-up staining with anti-IgG1 and anti-IgG2a-specific antibodies labeled with different fluorochromes. Similarly, if one of the primary antibodies is an IgM class and the other is IgG, then detection may be accomplished with specific anti-IgM and IgG labeled with different fluorochromes. The key is the narrow specificity of the second antibody and the design of appropriate controls to verify no crossreactivity of the reagents with the alternate targets (i.e., the anti IgG2a indeed does not react with the IgG1 primary antibody and vice versa). Another factor that must be considered when using indirect staining procedures to detect intracellular antigens followed with a direct label for a surface marker is the stability of the surface marker to the fixation/permeabilization procedure.

2. The use of fluorochrome-labeled control antibodies of the same isotype, or labeled preimmune serum for polyclonal reagents is important for flow cytometric analysis of intracellular as well as surface marker studies. The control reagents should be used at the same concentration as the specific antibodies. Such controls are particularly important when studying antigens that are present at low densities, resulting in fluorescence distributions that trail off the level of the negative control—reagent/antigen combinations that give clearly resolved positive and negative patterns are not as critical.
3. Compensation for spectral overlap of the different fluorochromes used in multicolor flow cytometry is addressed in Chapters 1 and 2. In our experience, using the methods described in this chapter, compensation settings that are effective for surface marker studies work fairly well for intracellular antigen studies. The most common need for adjustment occurs when staining antigens with high levels of expression with excess overlap of FITC (FL1) into the PE (FL2) channel and occasionally similar problems with excess FL2 signal in the FL3 channel using a dye emitting in the 670-nm range. Gating strategies may include standard FWD vs SSC gating since scatter characteristics are preserved. Alternatively, gating may be performed based on "immunoscatter" *(15,16)* in which one of the fluorescence parameters is plotted against one of the scatter parameters. We have successfully utilized fluorescence vs SSC with parameters such as CD19 FL3 vs SSC for intracellular light-chain or TdT analysis and CD38 vs SSC to gate plasma cells for light- or heavy-chain studies.
4. One problem that may occur with flow cytometric immunofluorescence analysis of either surface or intracellular markers is the potential for high background or nonspecific binding of the antibody to the cells. If autofluorescence is detected,

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Detection of Cyclins in Individual Cells by Flow and Laser Scanning Cytometry

Gloria Juan and Zbigniew Darzynkiewicz

1. Introduction

Progression of cells through successive phases and checkpoints of the cell cycle is maintained by sequential phosphorylation of different sets of nuclear and cytoplasmic proteins by cyclin-dependent kinases (CDKs) *(1–12)*. By activating their partner CDKs and targeting them to the respective protein substrates cyclins play a key regulatory role in this process. Cyclins B1, A, E, and D are expressed discontinuously during the cycle. The synthesis and degradation of these cyclins occurs at well-defined time points of the cell cycle (**Table 1**).

Cyclin B1 activates CDC2 whose kinase activity is essential for cell transition from G2 to M *(6,11)*. The onset of cyclin B1 accumulation is seen at the time of cell exit from S. Maximal levels of cyclin B1 exhibit cells entering mitosis. This cyclin is degraded rapidly during the transition to anaphase *(13)*. Cyclin A associates with either CDC2 or CDK2; the kinase activity of the complex drives the cell through S and G2 phases of the cycle *(14–16)*. Cellular accumulation of cyclin A starts early in S and its maximal expression is seen at the end of G2. This protein is rapidly degraded in prometaphase and the metaphase cells are essentially cyclin A negative. The kinase partner of cyclin E is CDK2 and this holoenzyme is essential for cell transition from G1 to S phase. Cyclin E starts to accumulate in the cell in mid-G1 and is maximally expressed at the time of cell entrance to S. Its continuous breakdown takes place as the cell progresses through S *(17–19)*. Expression of the different members of D family of cyclins (D1, D2, and D3) is tissue and cell-type specific. These cyclins are maximally expressed in response to mitogenic stimulation of G0 cells or by mitogens and growth factors. During exponential phase of cell growth their level appears to decrease *(20–25)*. Cyclins D activate

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Darzynkiewicz

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, St. Louis, MO).

4. Cell fixative: In 80% ethanol. Keep in the freezer (approx –20°C) prior to use.
5. RNase A (DNase-free RNase, available from Sigma).
6. 1 mg/mL RNase A in phosphate-buffered saline (PBS) (DNase-free RNase, available from Sigma).
7. 1% bovine serum albumin (BSA) in PBS.
8. 0.25% Triton X-100 in PBS.

3. Methods

3.1. Cyclin Detection by Flow Cytometry

1. Fix cells in suspension by pipeting 1–2 × 10^6 cells in 0.5 mL of PBS (phosphate buffered salt solution). Add 5 mL of ice-cold 80% ethanol (cyclins E, A, and B1) or ice-cold 100% methanol (D-type cyclins). The cells may be stored in this fixative in the cold (–20 to –40°C) for 2–24 h (*see* **Note 1**).
2. Centrifuge cells at 300*g* for 5 min. Resuspend cell pellet in 5 mL PBS. Keep for 5 min at room temperature. Spin at 300*g* for 5 min. Repeat once more with PBS containing 1% bovine serum albumin (BSA), centrifuge again, and suspend the cell pellet (<10^6 cells) in 1 mL 0.25% solution of Triton X-100 in PBS. Keep on ice for 5 min. Add 5 mL PBS. Centrifuge at 300*g* for 5 min.
3. Suspend cell pellet in 100 µL 1% BSA in PBS containing the primary antibody at the appropriate dilution to obtain 0.5 µg of antibody per sample. Incubate for 60 min at room temperature with gentle agitation or at 4°C overnight. Control cells should be treated identically; however, the control cells should be incubated with the same batch of isotypic antibody instead of a cyclin antibody (*see* **Note 3**).
4. Rinse the cells with 1% BSA in PBS, centrifuge at 300*g* for 5 min.
5. Suspend the cell pellet in 100 µL 1% BSA in PBS containing FITC-conjugated goat anti-mouse IgG antibody (diluted 1:30). Incubate for 30 min in the dark at room temperature with gentle agitation.
6. Rinse the cells with 1% BSA in PBS; centrifuge at 300*g* for 5 min.
7. Suspend the cell pellet in a solution containing 5 µg PI and 1 mg/mL of DNase-free RNase A in PBS. Incubate 20 min at room temperature in the dark before measurement.
8. Measure cell green (530+20 nm; FITC) and red (>590 nm; PI) fluorescence on the flow cytometer using blue light excitation (argon laser).

3.2. Cyclin Detection by Laser-Scanning Cytometer

The method described above can be adapted to stain cells mounted on microscope slides, to be analyzed by multiparameter LSC. To be analyzed by Laser scanning cytometer (LSC) the cells are initially attached to the slides by cytospinning, fixed, rinsed, and then subjected to the procedures as presented above (**Subheading 3.1.**).

1. To attach cells by cytospinning, 300 µL of suspension in tissue culture medium (with serum) containing approx 20,000 cells are added into a cytospin chamber.